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STUDIES ON THE CLEARANCE OF RADIOIODINATED PERTUSSIS VACCINE FROM THE LUNGS OF IMMUNIZED AND NON-IMMUNIZED MICE FOLLOWING INTRATRACHEAL ADMINISTRATION[1]

J. E. LOGAN, B. W. GRIFFITHS, AND M. A. MASON

Abstract

The intratracheal route of administration has been shown by means of radioiodinated pertussis vaccine to give more reproducible deliveries to the lung tissue in the challenge of mice than the intranasal route. Mice immunized 1 day or longer with a single immunizing dose of pertussis vaccine cleared a significantly higher amount of radioactivity from their lungs for a period of 24 hours following the intratracheal administration of radioiodinated pertussis vaccine than did non-immunized mice. Among those animals that received the labeled vaccine intratracheally, the mice that were immunized 6 days to 30 days previously showed higher fresh weights of lungs than those not immunized. The significance of these results is discussed.

Introduction

In a previous report from this laboratory (6), using I^{131}-labeled pertussis vaccine and challenging intranasally, considerable variation in the delivery of numbers of organisms to the lungs of individual animals was noted. Despite this variation, evidence was obtained which showed that lungs from mice previously immunized by a single injection of pertussis vaccine cleared the labeled vaccine more rapidly than lungs from non-immunized mice. To study this latter phenomenon more closely, and in order to overcome the handicap posed by the marked variation in numbers of bacteria reaching the lungs of the different animals, a technique of intratracheal challenge was applied. This paper presents the results obtained using this technique.

Methods

Mice

White mice,* 19 to 27 g. in weight, were used. Animals of the same sex and of similar age and weight as those immunized were used as the non-immunized controls. The mice were immunized by the intraperitoneal route with 0.5 ml. of a 1:4 dilution of pertussis vaccine† which was standardized against the World Health Organization Opacity Standard to contain approximately 2.4×10^9 organisms/ml.

[1]Manuscript received February 26, 1958.
Contribution from the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

*Strain bred by Connaught Medical Research Laboratories, University of Toronto.
†Supplied by Connaught Medical Research Laboratories, University of Toronto.

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Challenge Procedures

(*a*) *The intratracheal route.*—An adaptation of Bradford's technique (3) was used for the intratracheal challenge. A longitudinal ventral incision was made in the neck of the mouse under nembutal anesthesia. The trachea was exposed and 0.05 ml. of pertussis vaccine labeled with I^{131} (5) was introduced using a 0.25 ml. syringe and a No. 26 needle. Surgical clips were used to close the incision. The vaccine used was concentrated sixfold prior to the iodination in order to increase its specific activity. This resulted in a concentration of approximately 146×10^9 organisms/ml. in the challenge dose.

(*b*) *The intranasal route.*—The technique followed was described previously (6).

Removal of Tissues

At the time of killing, the mice were exsanguinated and the lungs removed, cleaned of adhering tissue, and placed on tared planchettes to obtain fresh weights.

Radioactivity Measurements

To control the dosage one standard delivery of 0.05 ml. from the syringe was made onto a planchette for each five animals injected intratracheally. The mean count of a series of deliveries of the labeled vaccine was taken as the dose administered. Radioactivity measurements of the lungs were made with a Nuclear Chicago Model DS-1 Scintillation counter and a Model 192 Ultrascaler. Corrections for iodine-131 decay were applied on all counts and the results were expressed as percentages of the administered dose.

Results

Intranasal vs. Intratracheal Route of Administration

Table I shows some comparative figures on the recovery of radioiodinated pertussis vaccine in the lungs of mice following administration by the intranasal and intratracheal routes. The recovery of labeled material from the

TABLE I

COMPARISON OF RECOVERY OF RADIOIODINATED PERTUSSIS VACCINE IN THE LUNGS OF MICE FOLLOWING ADMINISTRATION BY THE INTRANASAL AND INTRATRACHEAL ROUTES

Time after I^{131}-PV injection	Percentage of administered dose ± S.E.M.	
	Intranasal	Intratracheal
10 minutes:		
Non-immunized	52.3±4.4 (18)*	96.7±1.25 (19)
24 hours:		
Non-immunized	9.37±1.17 (19)	26.4±1.66 (14)
Immunized	4.64±0.78 (20)	18.0±0.68 (16)
	P value <0.01	*P* value <0.001

*Figures in parentheses denote the number of mice.

lungs both at 10 minutes and at 24 hours after administration was higher following intratracheal instillation. It will also be noted that the standard errors relative to the mean values were lower for the intratracheal groups.

Time After Intratracheal Administration

Table II shows the changes in the fresh weights of the lungs of 20-day immunized and non-immunized mice at 10 minutes, 2, 6, 24, and 48 hours following the intratracheal administration of radioiodinated pertussis vaccine. The fresh weights of these lungs have been corrected to that of 20-g. animals in each case in order to form a better comparison between groups of mice with differing body weights. This was done by first setting up a scale of lung-weight data obtained from groups of normal mice of different mean body weights. It will be noted in Table II that the fresh weights of the lungs of both the immunized and non-immunized mice were higher at 10 minutes than they were at 2 hours following the injection. However, in both groups there was a continuous gain in weights of lungs from 2 to 48 hours. This weight gain was greater in the immunized mice than in the non-immunized animals in the 6-hour to 24-hour period. The differences in fresh weights of lungs between these two groups were statistically significant at 24 and 48 hours after the injection.

TABLE II

EFFECT OF TIME FOLLOWING INTRATRACHEAL ADMINISTRATION OF RADIOIODINATED PERTUSSIS VACCINE ON THE FRESH WEIGHTS OF LUNGS OF NON-IMMUNIZED MICE AND MICE IMMUNIZED 20 DAYS PREVIOUSLY WITH PERTUSSIS VACCINE

Time	Mean fresh wt. ± S.E.M.,† mg.		*P* values
	Immunized	Non-immunized	
Uninjected	166± 6 (23)*	161± 5 (20)	$P > 0.5$
10 minutes	214± 8 (25)	211±11 (19)	$P > 0.8$
2 hours	188± 7 (17)	178± 8 (21)	$P > 0.3$
6 hours	205± 7 (16)	206±10 (19)	$P > 0.9$
24 hours	264± 3 (175)	228± 3 (160)	$P < 0.001$
48 hours	279±10 (18)	243± 7 (18)	$P < 0.01$

*Figures in parentheses denote the number of mice.
†Corrected to 20 g. body weight.

The results of the radioactivity measurements in the lungs of these same animals are shown in Table III. In both immunized and non-immunized mice, the level of radioactivity after challenge showed a steady decline up to the 48-hour period. Two hours after challenge the lungs of the immunized mice were more radioactive than those of the normal animals but at 24 and 48 hours following the intratracheal administration, the levels in the lungs of the immunized mice were significantly lower than those of the non-immunized controls.

TABLE III

EFFECT OF TIME FOLLOWING INTRATRACHEAL ADMINISTRATION OF RADIOIODINATED PERTUSSIS VACCINE ON THE CLEARANCE OF LABELED MATERIAL FROM THE LUNGS OF NON-IMMUNIZED MICE AND MICE IMMUNIZED 20 DAYS PREVIOUSLY WITH PERTUSSIS VACCINE

Time	Percentage of administered dose ± S.E.M.		*P* values
	Immunized	Non-immunized	
10 minutes	96.9 ±1.18 (25)*	96.7 ±1.25 (19)	$P > 0.9$
2 hours	78.2 ±1.06 (17)	74.3 ±1.51 (21)	$P < 0.05$
6 hours	50.2 ±1.32 (16)	51.7 ±1.20 (19)	$P > 0.4$
24 hours	15.5 ±0.46 (116)	19.2 ±0.63 (101)	$P < 0.001$
48 hours	6.05±0.33 (18)	7.65±0.37 (18)	$P < 0.01$

*Figures in parentheses denote the number of mice.

The Effect of the Length of the Immunization Period Prior to Challenge

Table IV shows the differences in fresh weight (corrected to 20 g. body weight) of lungs between groups of normal untreated mice and groups of immune and non-immune mice that had received the radioactive challenge 24 hours prior to killing. It will be noted that all intratracheally injected mice, immunized or not, had higher lung weights than those of the uninjected animals. However, in the case of mice immunized from 6 to 30 days prior to the injection these higher lung weights were increased still further to figures significantly above those of the non-immunized mice. This difference was not evident in the lungs of animals 1 day after immunization and was no longer apparent 98 days after immunization.

Table V shows the amounts of radioactivity remaining 24 hours after intratracheal challenge in the lungs of non-immunized mice and of mice immunized for varying periods of time. All mice except the 98-day immunized group

TABLE IV

EFFECT OF VARYING THE TIME OF IMMUNIZATION ON THE FRESH WEIGHTS OF LUNGS OF MICE AT 24 HOURS FOLLOWING INTRATRACHEAL ADMINISTRATION OF RADIOIODINATED PERTUSSIS VACCINE

Days of immunization	Mean fresh wt. ± S.E.M.,† mg.		*P* values
	Immunized	Non-immunized	
1	254±10 (51)*	243±7 (41)	$P > 0.3$
6	259± 3 (212)	231±3 (223)	$P < 0.001$
9	269± 6 (16)	237±7 (14)	$P < 0.001$
14	266±10 (18)	231±8 (17)	$P < 0.01$
20	264± 3 (175)	228±3 (160)	$P < 0.001$
30	256± 6 (47)	226±5 (48)	$P < 0.001$
98 (multiple)	216± 5 (58)	228±7 (20)	$P > 0.1$
Uninjected controls	166± 6 (23)	161±5 (20)	$P > 0.5$

*Figures in parentheses denote the number of mice.
†Corrected to 20 g. body weight.

received a single immunizing dose of pertussis vaccine. The 98-day immunized group received eight immunizing doses spaced a week apart. The final dose in this group was given 7 weeks before the intratracheal challenge. In all cases, whether immunized 1 day or 98 days earlier, the immunized mice had lower levels of radioactivity in their lungs than the non-immunized controls. These differences were highly significant statistically.

TABLE V

EFFECT OF VARYING THE TIME OF IMMUNIZATION ON THE CLEARANCE OF RADIOIODINATED PERTUSSIS VACCINE FROM THE LUNGS OF MICE AT 24 HOURS FOLLOWING ITS INTRATRACHEAL ADMINISTRATION

Days of immunization	Percentage of administered dose ± S.E.M.		*P* values
	Immunized	Non-immunized	
1	14.7±0.59 (51)*	18.0±0.60 (41)	$P < 0.001$
6	16.0±0.42 (88)	20.2±0.49 (93)	$P < 0.001$
9	18.0±0.08 (16)	26.4±1.66 (14)	$P < 0.001$
14	18.4±0.64 (17)	22.0±0.83 (17)	$P < 0.01$
20	15.5±0.46 (116)	19.2±0.63 (101)	$P < 0.001$
30	17.7±0.93 (40)	23.5±1.18 (42)	$P < 0.01$
98 (multiple)	11.4±0.43 (61)	16.3±0.57 (20)	$P < 0.001$

*Figures in parentheses denote the number of mice.

Discussion

A comparison of the intranasal and intratracheal routes for the administration of radioiodinated pertussis vaccine has shown both methods to have advantages and disadvantages. The intratracheal route has been shown to be the more useful, since it allows for a significantly higher uptake in the lungs than the intranasal route and in addition has a much smaller variation of uptake between lungs. The intranasal challenge may be administered more easily and speedily but these advantages are somewhat offset by the fact that one quarter to one third of the challenged mice reject some portion of the administered dose and have to be replaced. The loss of mice following intratracheal challenge due to minor surgery and dose rejection is about 10 to 12%.

Our results have shown that the fresh weights of the lungs of all mice, whether immunized or not, receiving the intratracheal challenge of radioiodinated pertussis vaccine were higher than those of mice not receiving the injection. The higher fresh weights recorded at 10 minutes following challenge were undoubtedly due to the weight of the challenge dose which was approximately 50 mg. Twenty-four hours after challenge the lungs from mice that were immunized 6 days to 30 days prior to challenge were found to be heavier than those of non-immunized mice. Gross examination of the lungs of the challenged mice revealed considerable inflammation suggesting that the increased weight could have been due to edema. From this, it would follow that lungs of mice immunized 6 days or longer have a higher edema tendency. This was supported by subsequent histological examination of lung sections of immunized and non-immunized mice. A greater degree of

hyperemia and a marked infiltration of blood cells were found in the lung tissue of the immunized animals. We have not established the factor or factors responsible for the production of this lung edema, but a consideration of the results obtained is interesting in light of the observations of other workers. Andersen (2) found that the lung weights of mice vaccinated 14 days prior to an intranasal challenge with a sterile disintegrated cell suspension of *Hemophilus pertussis* were greater than those of similarly treated non-immunized mice. Her results were based on weights recorded 7 days after intranasal inoculation as compared to the shorter intervals of 24 and 48 hours recorded in our experiments. She suggested that the previous pertussis vaccination may have caused development of the lung edema because of the action of its histamine-sensitizing component. Maitland, Kohn, and MacDonald (7) showed that the histamine sensitivity produced by pertussis vaccine reached a maximum in 3–4 days which was maintained for about 2 weeks and then gradually disappeared during the next 2–3 weeks. Recently, Munoz (8) described a sensitivity of mice to serotonin following injection of *H. pertussis* cells. This sensitivity could be detected in 16 to 24 hours following administration, reached its peak on the third to fourth day, and disappeared by the 25th day.

Our findings would indicate that the lung edema effect produced by pertussis vaccine may not be completely attributable to histamine and/or serotonin-sensitizing factors. Firstly, the fresh weights of lungs of mice immunized 30 days previously were found to be significantly heavier than those of non-immunized controls, at a time when the sensitivity to either histamine or serotonin has largely disappeared. Secondly, we found that the fresh weights of the lungs of mice immunized 6 days earlier with pertussis vaccine, heated for 30 minutes at 80° C. to destroy the histamine-sensitizing factor (7), were significantly higher than those from non-immunized controls. They were, however, not as heavy as lungs from mice treated with unheated vaccine. Histamine injection of an identical group of mice immunized with heated vaccine confirmed that this heating procedure reduced the histamine-sensitizing capacity of the vaccine. These results indicate that a factor or factors additional to histamine and/or serotonin-sensitizing factors and which are not destroyed by heating at 80° C. for 30 minutes are contributing to the higher lung weights observed in the immunized mice. Parfentjev, Goodline, and Virion (9) described a sensitization to *H. pertussis* protein antigen following pertussis vaccine immunization which reaches a peak in 11–12 days and which persisted for several months. A sensitizing factor such as this whose effects apparently last longer may also be affecting the lung weights particularly in the case of the mice immunized for 30 days.

In these studies, it was found that 24 hours after intratracheal challenge with radioiodinated pertussis vaccine, mice immunized with a single dose 1 or more days earlier were able to clear a significantly higher amount of the challenge from their lungs than non-immunized mice. This accelerated clearance is probably due to phagocytosis, which is known to be enhanced by specific antibodies. The explanation for the greater clearance noted in the lungs of

mice immunized for only relatively short periods such as 1 day and 6 days is not so clear since it occurred prior to the detection of circulating antibodies. Evans and Perkins (4) demonstrated an interference type of immunity present in mice within a few hours after immunization and which they postulated reached its maximum effect in about 10 days and then disappeared. Andersen (1) questioned these findings, pointing out that it is possible to demonstrate local antibody formation within a few hours after vaccination.

Sternberg *et al.* (10, 11, 12) postulated that the faster clearance of iodine-131 and other radioisotopes was due to a state resembling hyperthyroidism in immunized animals. They observed a two-stage stimulation of turnover of radioisotopes, the first at the start when immunity has not been established and the second at the time of peak immunization of the animal. They felt that an increased utilization of hormones at the tissue level such as occurs in hyperthyroidism might be responsible for a generally higher metabolic rate and hence a more rapid turnover of radioisotopes. If these contentions were true, the injection of non-specific antigens might be expected to accelerate the clearance and breakdown of iodinated vaccine but this has not been borne out in our studies where several other antigens were tried.

Further work is in progress to try and determine the nature of this clearance phenomenon in mice in relation to immunity against *H. pertussis*.

Acknowledgments

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THE HEXOSEMONOPHOSPHATE PATHWAY IN ASPERGILLUS NIGER[1]

M. W. McDonough and S. M. Martin

Abstract

Enzymes of the hexosemonophosphate pathway were demonstrated in extracts obtained from *Aspergillus niger* grown in two types of media: a fermentation medium (molasses) and a rich growth medium (MYG). Preparations from cells grown in either medium contained a TPN-specific glucose-6-phosphate (G-6-P) dehydrogenase and a TPN-linked 6-phosphogluconate (6-P-G) dehydrogenase. The rate of pyridine nucleotide reduction by the latter was 20% of that by G-6-P dehydrogenase. For both enzymes the activity of the preparation from molasses cultures was twice that from MYG cultures. A DPN-linked 6-P-G dehydrogenase was demonstrated in molasses preparations though not in MYG preparations. On incubating extracts with R-5-P aerobically, total pentose decreased accompanied by a sequential increase and decrease in ketopentose. An increase in hexose was observed under anaerobic conditions but accounted for only one third of the pentose. Increases in heptulose and triose occurred only in the presence of hydrazine. The presence of phosphohexoisomerase, which is necessary to complete a "pentose cycle", was demonstrated.

Introduction

Available evidence has demonstrated that many molds can dissimilate glucose in several ways. Heath, Nasser, and Koffler (6) suggested, on the basis of experiments using labeled substrate, that *Fusarium lini* utilized the glycolytic pathway under anaerobic conditions, whereas under aerobic conditions metabolism was oxidative. Heath and Koffler (5), using growing cells of *Penicillium chrysogenum*, concluded that about two thirds of the glucose was metabolized oxidatively, presumably via the hexosemonophosphate pathway. It was also shown (5) that incubation of extracts of *P. chrysogenum* with R-5-P* led to the disappearance of pentose. The enzymes of the glycolytic scheme, except phosphohexokinase, were demonstrated and hexose, pentose, and heptose phosphates were isolated from *P. chrysogenum* by Sih and Knight (16).

In a study of citrate formation by *Aspergillus niger*, Shu, Funk, and Neish (14) concluded that nearly 80% of the glucose was dissimilated via the glycolytic pathway, the remainder being degraded by some other mechanism. Jagannathan and Singh (7) demonstrated the enzymes of glycolysis in extracts of *A. niger* and Damodaran, Jagannathan, and Singh (1) studied the quantitative changes in these enzymes during the fermentation. The latter also studied the G-6-P and 6-P-G dehydrogenases, two enzymes which initiate another route of glucose metabolism. Van Sumere and Shu (18) concluded,

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*Abbreviations used: DPN, diphosphopyridine nucleotide; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconate; R-5-P, ribose-5-phosphate; TPN, triphosphopyridine nucleotide.

from a study of the biosynthesis of mycelial glucan by *A. niger* from labeled substrates, that pentoses are converted to glucose via transaldolase and isomerase reactions. Thus although the evidence strongly supported the occurrence of the hexosemonophosphate pathway in *A. niger*, demonstration of the enzymes concerned was lacking. Work was therefore initiated to investigate the presence of these enzymes in extracts of *A. niger*.

Materials and Methods

A. niger, N.R.C. A_1-233, was grown at 28° C. in shake flasks containing 200 ml. of medium. Two quite different media were used: malt extract – yeast extract – glucose (MYG) (8) and ferrocyanide-treated beet molasses (molasses) (17). The mycelium from 24-hour MYG cultures or 36-hour molasses cultures was removed by filtration, washed several times with water, and filtered on a buchner funnel. The yield from MYG cultures was 20 to 25 g. wet weight of mycelium, while that from molasses cultures was 15 to 20 g. Extracts were prepared by grinding 18 g. of mycelium in a porcelain planetary ball-mill with 18 g. of acid-washed quartz grit and 27 ml. of 0.1 *M* phosphate buffer, pH 7.0. Cell debris and quartz were removed by centrifugation at about $2000 \times g$ for 30 minutes followed by centrifugation at about $18{,}000 \times g$ for 20 minutes, both at 0° C.

Commercial preparations of G-6-P, R-5-P, F-6-P, DPN, and TPN were employed. 6-Phosphogluconate was prepared according to the method of Seegmiller and Horecker (13) except that a 16-hour oxidation period was used. The barium salts of the phosphate esters were converted to the sodium salts by treatment with sodium sulphate. The purity of substrates was checked by chromatographic and electrophoretic methods.

The activity of the enzymes concerned with the hexosemonophosphate pathway were demonstrated using crude extracts prepared as above. Reduction of DPN and TPN was followed at 349 mμ with a Cary recording spectrophotometer. Protein was estimated by the spectrophotometric method of Warburg and Christian (19). Total hexose was determined by the method of Nelson (11), total pentose by the method of Mejbaum (10), ketohexose by the method of Dische and Borenfreund (3), heptose by the method of Dische (2), and triose by the method of Sibley and Lehninger (15). Heat-inactivated controls were included in all experiments.

Results

Hexose phosphate is converted to pentose phosphate by the action of G-6-P and 6-P-G dehydrogenases. Extracts prepared from cells grown in either molasses of MYG contained an active TPN-specific G-6-P dehydrogenase. However, as shown in Fig. 1, the activity of the preparation from molasses cultures was twice that of the preparation from MYG cultures. A DPN-linked G-6-P dehydrogenase was not demonstrable in either preparation. Both preparations also contained a TPN-linked 6-P-G dehydrogenase, but the rate of reduction of cofactor was only about 20% of that when G-6-P was the substrate. Once again molasses preparations were about twice as active as

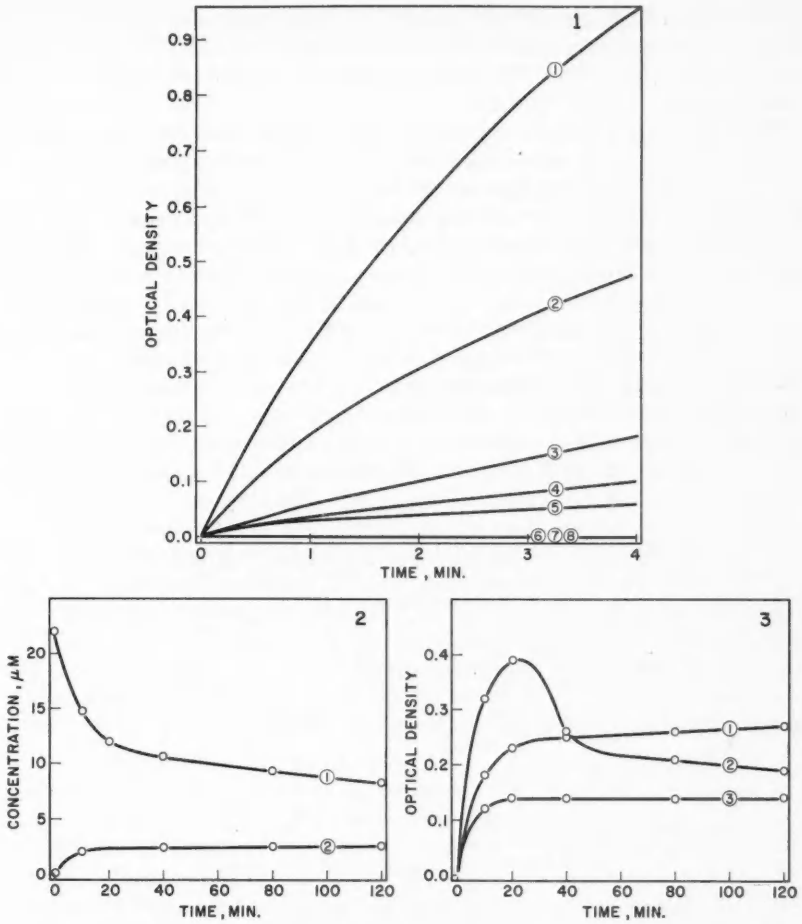

FIG. 1. G-6-P and 6-P-G dehydrogenases. Cuvettes contained 1.6 mg. of protein and 0.5 μM. of DPN or TPN in 2.8 ml. of water. Reaction was started by adding 10 μM. of substrate in 0.2 ml. of water. 1 = molasses prep. + TPN + G-6-P; 2 = MYG prep. + TPN + G-6-P; 3 = molasses prep. + TPN + 6-P-G; 4 = MYG prep. + TPN + 6-P-G; 5 = molasses prep. + DPN + 6-P-G; 6 = molasses prep. + DPN + G-6-P; 7 = MYG prep. + DPN + G-6-P; 8 = MYG prep. + DPN + 6-P-G.

FIG. 2. Pentose (1) utilization and hexose (2) formation under anaerobic conditions. Reaction started by tipping 25 μM. R-5-P in 0.5 ml. of water into 12 mg. of protein in 2.5 ml. of water. Atmosphere = nitrogen. Temperature = 27° C. Flasks removed at times indicated and assayed for pentose and hexose.

FIG. 3. Heptulose (1), ketopentose (2), and triose (3) formation under aerobic conditions. Ketopentose: reaction started by tipping 25 μM. R-5-P in 0.5 ml. of water into 12 mg. of protein in 2.5 ml. of water; 0.2 ml. aliquots removed at intervals and assayed for ketopentose. Heptulose and triose: 50 μM. R-5-P in 1.0 ml. of water tipped into 24 mg. of protein + 99 μM. neutral hydrazine sulphate in 5 ml. of water; pairs of 0.5 ml. aliquots removed at intervals and assayed for heptulose and triose. Atmosphere = air. Temperature = 27° C.

Because of lack of pure reference compounds determination of amounts of products present was not possible and thus O.D. values are reported.

MYG preparations. A DPN-linked 6-P-G dehydrogenase was also demonstrable in molasses preparations with the rate of reduction of DPN being about 30% of that with TPN. This latter dehydrogenase was not found in MYG preparations.

The regeneration of hexose phosphate from pentose phosphate via heptulose phosphate is through transketolase and transaldolase reactions. When extracts prepared from molasses or MYG cultures were incubated aerobically with R-5-P, a decrease in pentose resulted. For example, with a molasses preparation the rate of pentose utilization was 7.5 μM. per mg. of protein per hour measured over the first 10 minutes of reaction time, whereas with an MYG preparation the rate was 4.5 μM. Since extracts prepared from molasses-grown cells were nearly twice as active as those from MYG-grown cells they were used in subsequent trials. Under anaerobic conditions, the decrease in pentose was accompanied by an increase in hexose (Fig. 2). In the example cited 16.9 μM. of pentose disappeared and 2.6 μM. of hexose appeared. Since neither heptulose nor triose could be detected in the reaction mixture they could not account for the missing 11.7 μM. of pentose. Under aerobic conditions total pentose decreased, accompanied by a sequential appearance and disappearance of ketopentose (Fig. 3). An increase in hexose was not observed and neither heptulose nor triose was detectable by colorimetric tests. In the presence of hydrazine, however, both heptulose and triose were shown to increase (Fig. 3). The final reaction in the pentose cycle, the regeneration of G-6-P, is catalyzed by phosphohexoisomerase. The presence of this enzyme was demonstrated by incubating under anaerobic conditions either F-6-P or G-6-P with extracts prepared from molasses cultures and determining the levels of fructose and total hexose after 4 hours' incubation (Table I).

TABLE I

PHOSPHOHEXOISOMERASE

	Total hexose, μM.	Fructose, μM.	Glucose,* μM.	Glucose/fructose
Substrate = G-6-P				
Control	3.62	0	3.62	–
Experimental	3.62	0.88	2.74	3.12
Substrate = F-6-P				
Control	3.73	3.50	0.23	–
Experimental	3.73	1.00	2.73	2.73

*Obtained by difference; 0.1 ml. of substrate was tipped into 0.5 ml. of crude extract (or heat-inactivated extract) and incubated at 27° C. for 4 hours, atmosphere = N_2.

Discussion

Undoubtedly a number of different mechanisms for the dissimilation of carbohydrates exist in *A. niger*. The present demonstration of the reactions of the hexosemonophosphate oxidative pathway, together with the supporting data obtained using tracers (18), indicates that this scheme is operative in *A. niger*. Demonstration of the enzymes of the glycolytic pathway (7)

together with the supporting data (14) indicates that that mechanism is also operative. It is also known that this organism is capable of producing large amounts of gluconate and ketogluconate (9) and these may represent products of the beginning reactions of a third route. Probably other mechanisms also exist since this organism has very simple nutritional requirements and can metabolize a wide variety of carbohydrates. One pathway which would be attractive from the point of citrate production because of the conservation of carbon is the Entner–Doudoroff (4) scheme for the breakdown of 6-P-G to pyruvate and glyceraldehyde-3-phosphate. However, in the present study we were unable to detect the required enzymes in extracts from cells grown under a variety of conditions.

Since it is well known that the metabolism of molds varies greatly with cultural conditions, it seems reasonable to assume that the relative importance of the two established pathways in the metabolism of the organism will also vary with conditions. This probably accounts for the differences in activity toward intermediates of the pentose cycle which were observed with extracts prepared from cells grown on the two widely different media used in this study.

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SYNTHESIS OF ASPARTIC ACID BY LACTOBACILLUS ARABINOSUS[1]

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Abstract

Lactobacillus arabinosus was grown on a complex synthetic medium and the synthesis of aspartic acid studied using the tracer compounds carbon dioxide-C^{14}, glucose-6-C^{14}, glucose-2-C^{14}, or ammonium chloride-N^{15}. The β-carboxyl carbon of aspartic acid was derived to a major extent or solely from carbon dioxide-C^{14} and no radioactivity was found in the α-carboxyl. Approximately half of the β-carbon atoms of aspartic acid were derived from carbon 6 of glucose and half of the α-carbon atoms from carbon 2 of glucose. The amino group of aspartic acid was derived to only a minor extent from ammonium chloride-N^{15} and the isotope concentration was the same as that found in glutamic acid. The results indicate that aspartic acid is formed by the carboxylation of two three-carbon fragments derived from glucose, followed by transamination.

Malic acid or oxalacetic acid added to the medium was not used to a significant extent in the synthesis of aspartic acid. The addition of aspartic acid to the medium caused a net decrease in the amount of aspartic acid synthesized, and the effect was more marked in low-biotin medium.

Introduction

Lactobacillus arabinosus and some other lactic acid bacteria have been shown to synthesize aspartic acid when grown on media containing no aspartic acid (28). Both biotin and carbon dioxide have been shown to be important in this synthesis (2, 12, 14), which is one of the few metabolic reactions in which biotin has been implicated. Some direct evidence as to the pathway of aspartic acid synthesis in *L. arabinosus* has been obtained by Lardy *et al.* (11), who found that cell suspensions of the organism fixed carbon dioxide-C^{14} into either or both the carboxyl groups of aspartic acid. However, the exact location of the isotope in aspartic acid and the source of the other carbon atoms in the molecule were not determined, and it was not possible to say whether carbon dioxide was the sole or partial source of carbon for one or both the carboxyl groups of aspartic acid. Indirect evidence as to the pathway of aspartic acid synthesis has been supplied by Potter *et al.* (22), who concluded from growth experiments with *L. arabinosus* that oxalacetic acid is an intermediate in aspartic acid synthesis, and that the synthesis of oxalacetic acid is impaired by biotin deficiency. In the present work, the synthesis of aspartic acid by *L. arabinosus* was studied using carbon dioxide-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, and ammonium chloride-N^{15}. The effect of oxalacetic acid, biotin, and some other compounds on this synthesis was also investigated.

Experimental

Cultures of *L. arabinosus* 17-5 were maintained (2) on Difco microinoculum agar and broth (Difco Laboratories, Detroit, Michigan) supplemented with 0.01 ml. of salts B (22) per ml. of medium. In most experiments 50–60 ml.

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of synthetic medium (22), supplemented with 10^{-8} *M* biotin and 3 mg./ml. of ammonium chloride, was inoculated with a washed suspension (2) of *L. arabinosus* and incubated at 37° C. in an atmosphere of carbon dioxide until it was considered that growth had almost ceased, since there was little or no change in turbidity over a 1-hour period. In experiment 1 the organism was grown in a sealed tube in medium supplemented with sodium bicarbonate-C^{14}; carbonate-free sodium hydroxide was added at the end of the growth period to prevent loss of carbon dioxide before analysis for carbonate (21). In later experiments with carbon dioxide-C^{14}, the procedure was changed so that larger volumes of carbon dioxide-C^{14} could be used (approximately 18 millimoles containing 50 microcuries) to decrease the effect of dilution by metabolic carbon dioxide, and so that various compounds could be added aseptically during the experiment. Either 50 ml. of high-biotin (10^{-8} *M*) medium or 150 to 200 ml. of low-biotin medium was autoclaved in a 500-ml. round-bottom flask similar to the fermentation flask described by Neish (20), and the flask filled with carbon dioxide-C^{14} generated from barium carbonate-C^{14} using standard high vacuum techniques and liquid air cooling (4). The low-biotin media contained a concentration of biotin (10^{-10} *M* in experiment 14 and 5×10^{-10} *M* in experiments 6 and 11) which supported approximately one-third the growth as the same volume of high-biotin medium.

In experiments 2, 3, and 15, radioactive glucose was autoclaved and added to sterile glucose-free medium to give a concentration of 0.05 *M*.

In experiments 5, 7 to 10, 12, 13, and 15, the inoculum of *L. arabinosus* amounted to about two per cent of the final cell yield and growth was almost complete in 8 hours; the figures for the specific activity of the aspartic acid isolated in these experiments have been increased by about two per cent to correct for the dilution by the cellular aspartic acid in the inoculum. In the other experiments, the inoculum was much smaller and growth was almost complete in 11 to 23 hours.

After the organism had grown, the flask was cooled and in experiments 5 to 14, most of the radioactive carbon dioxide recovered by transfer on the vacuum line, and converted to barium carbonate. The cells were centrifuged out of the medium, washed with water, and extracted twice with 2 ml. of water for 5 minutes in a boiling-water bath to remove intracellular amino acids (7), cooled, and the cells centrifuged down. The centrifuged medium was freed of carbon dioxide-C^{14} as described by Lardy *et al.* (11). The major portion of the cells was hydrolyzed in a sealed tube with 8 ml. 6 *M* HCl at 100° C. for 24 hours. The hydrolyzate was dried *in vacuo* and chromatographed on a column of Dowex-2 acetate 30 cm. long and 1 cm. in diameter using 0.2 *M* acetate buffer, pH 3.5, as the eluant (18). This procedure separates glutamic and aspartic acids, which were desalted (27) and analyzed by the method of Troll and Cannan (29). In experiments using radioactive glucose the aspartic acid fraction had to be rechromatographed to remove a radioactive contaminant.

After dilution with carrier aspartic acid, the aspartic acid samples were degraded to obtain the isotope distribution in the molecule. The α- plus

β-carboxyl groups were degraded to carbon dioxide with ninhydrin (9) and the β-carboxyl group with *Clostridium welchii* (17). The α- and β-carbons were obtained as the 2,4-dinitrophenylhydrazone of acetaldehyde and the β-carbon as iodoform (6). In the latter case the published procedure had to be modified to obtain a significant yield: the acetaldehyde was distilled directly into a solution of excess sodium borohydride, redistilled, and the ethanol solution treated at 50° C. with stepwise additions of the I_2–KI solution until the reaction mixture showed a permanent brown color.

Carbonate samples were converted to carbon dioxide with sulphuric acid and other samples oxidized to carbon dioxide (3). Radioactivity was determined with an ionization chamber and vibrating reed electrometer (23); duplicate samples usually agreed within 3%. In experiment 4 ammonia was separated from the medium, or Kjeldahl digests of amino acids, by steam distillation of alkaline solutions, and N^{15} was determined (26).

Results and Discussion

In experiments 1 to 11 and experiment 15, *L. arabinosus* was grown in medium containing no aspartic acid, and a small inoculum used compared to the final cell population. Essentially all the aspartic acid found in the cells and medium, then, must be synthesized from constituents of the medium. Within experimental error, all the radioactive carbon dioxide fixed in aspartic acid in experiment 1 was found in the β-carboxyl group (Table I). Using the average of the initial and final specific activity of the radioactive carbonate for calculation, one finds that the specific activity of the β-carboxyl carbon of aspartic acid in this experiment is 102% of that of the carbonate. This indicates that within experimental error all of the β-carboxyl carbon was derived from carbon dioxide. The decrease in specific activity of the carbonate on growth of the cells is due in part to a net production of carbon dioxide by the cells, for the carbonate concentration in the medium increased from an initial value of 8.7 m*M* to 9.4 m*M* at the end of the experiment. A similar production of carbon dioxide by *L. arabinosus* was noted in the experiments of Lardy *et al.* (11). Experiments 5 and 6 (Table III) confirm the results of experiment 1.

TABLE I

ISOTOPE DISTRIBUTION IN ASPARTIC ACID ISOLATED FROM *L. arabinosus* GROWN IN THE PRESENCE OF VARIOUS ISOTOPIC COMPOUNDS

Expt.	Compound labeled	Specific activity*	Specific activity of aspartic acid	Per cent of radioactivity of aspartic acid found in:			
				α and β carboxyl	β carboxyl	α and β atoms	β atom
1	$NaHC^{14}O_2$	54.4, 40.2†	50.4	96.6	96.0		
2	Glucose-6-C^{14}	1.60	0.79	<1		95	102
3	Glucose-2-C^{14}	1.57	0.82			94	<1
4	$N^{15}H_4Cl$	64.5, 64.3†	5–7				

*Specific activity is expressed for C^{14} as microcuries per millimole, and for N^{15} as atom % excess.
†These figures represent the values at the beginning and end of the growth period, respectively.

The specific activity of the aspartic acid was close to that of the carbonate and essentially all the activity in aspartic acid was found in the β-carboxyl group (97% in experiment 5 and 102% in experiment 6). Even on low-biotin medium (experiment 6), the β-carboxyl carbon of aspartic acid synthesized is derived from carbon dioxide.

Potter *et al.* (22) postulated that aspartic acid is synthesized in *L. arabinosus* by the β-carboxylation of pyruvic acid followed by amination. Gibbs *et al.* (8) have shown that lactic acid (and by inference, pyruvic acid) is formed from glucose by various homofermentative lactic acid bacteria via the Embden–Meyerhof scheme of glycolysis. According to these schemes, then, glucose-6-C^{14} could form one molecule of pyruvic acid-3-C^{14} plus one molecule of unlabeled pyruvic acid, and these could be carboxylated and aminated to form one molecule of aspartic acid, labeled in the β-carbon atom, and one molecule of unlabeled aspartic acid. Thus the aspartic acid synthesized by this pathway would have half the specific activity of the glucose-6-C^{14} and be labeled in the β-carbon atom; this is essentially the result obtained in experiment 2, Table I. The aspartic acid formed by this pathway from glucose-2-C^{14} would have half the specific activity of the glucose and be labeled in the α-carbon atom, and this was essentially the result obtained in experiment 3, Table I. The author considers that the above evidence is sufficient to establish that the β-carboxyl group of aspartic acid synthesized by *L. arabinosus* is derived from carbon dioxide and the rest of the molecule from glucose via two three-carbon fragments. Experiment 15, Table III, shows that pyruvate added to the medium decreases the incorporation of radioactivity from glucose-6-C^{14} into aspartic acid. This indicates that pyruvic acid, or a compound derived from it, is probably used in the synthesis of aspartic acid.

In some preliminary experiments in this laboratory it was found that in biotin-containing media to which no aspartic acid or ammonium chloride was added, the growth of *L. arabinosus* was less than half that obtained when either of these compounds was added to the medium in concentrations of 1.0 m*M* and 3 mg./ml., respectively. It appeared possible that the ammonium ion in the medium was the source of the amino nitrogen in aspartic acid. However, in experiment 4, Table I, it was found that the concentration of N^{15} in the amino group of aspartic acid was much less than that of the ammonium chloride-N^{15} added to the medium, which excludes the possibility that the amino group of aspartic acid was derived to a major extent from ammonium ion in the medium. The N^{15} concentrations in cellular glutamic and aspartic acids in this experiment were the same, so the amino group in aspartic acid may well be derived by transamination with glutamic acid supplied in the medium. This explanation of the results receives support from the work of other investigators who found that cell-free extracts of *L. arabinosus* can transaminate α-ketoglutaric acid and aspartic acid (16) and that pyridoxine is important in the synthesis of aspartic acid by this organism (14). The reaction by which N^{15} from the ammonium chloride in the medium was incorporated in the amino group of glutamic acid is not known, but it could be involved in the glutamic acid racemase system found in this organism (19).

Table II shows the general distribution of radioactivity in the cells and medium for the same experiments reported in Table I. In experiments 2 and 3, the organism was incubated in an atmosphere of non-radioactive carbon dioxide. Although this carbon dioxide was not collected, analysis of the medium containing the cells at the beginning and end of the growth period showed that there was no detectable loss of radioactivity and therefore little, if any, carbon dioxide produced from carbons 2 or 6 of glucose. In experiment 1, 90% of the radioactivity in the sodium bicarbonate-C^{14} added is accounted for, and a small loss of carbon dioxide on the opening of the sealed tube might account for the remainder. In this experiment only about 4% of the radioactivity fixed in the medium was found in effluent fractions from Dowex-2 chromatograms that could contain aspartic acid, and the activity in these fractions was too small to work with conveniently. These results show that about half the total activity fixed from radioactive carbon dioxide was present in compounds other than aspartic acid. These results differ from those obtained by Lardy *et al.* (11) in shorter experiments with suspensions of *L. arabinosus*, for these workers found that essentially all the carbon dioxide fixed was incorporated in aspartic acid. However, aspartic acid is used in the synthesis of other amino acids, purines, and pyrimidines by this organism (25) and one would expect that in longer experiments with growing cells these compounds would be synthesized from aspartic acid. Experiments 1 and 5 to 14 in Table III show that aspartic acid contains a relatively constant fraction (0.65 to 0.86) of the total radioactivity fixed in the cells from carbon dioxide-C^{14}, even though the amount of synthesis of aspartic acid varies from very little (experiment 14) to close to the maximum (experiments 1 and 5 to 7). The fact that the total amount of carbon dioxide fixation in the cells parallels the amount of carbon dioxide fixed in aspartic acid could be explained if the radioactive compounds in the cell other than aspartic acid were derived from aspartic acid or intermediates in aspartic acid synthesis. In contrast, the ratio of carbon dioxide-C^{14} fixed in the supernate to that fixed in the cells varies greatly from one experiment to another, and is so different in experiments which should be duplicates (experiments 12 and 13) that no postulate as to the pathways of synthesis of the radioactive compounds in the supernate can be made.

TABLE II

ISOTOPE DISTRIBUTION AFTER GROWTH OF *L. arabinosus* IN MEDIUM CONTAINING VARIOUS ISOTOPIC COMPOUNDS

Expt.	Compound labeled	Yield *L. arabinosus*, mg. dry weight/ml. media	Per cent of total activity found in:				
			CO_2	Centrifuged medium	Cell pool*	Cells	Cellular aspartate
1	$NaHC^{14}O_3$	0.82	80.5	2.8	0.3	6.3	5.0
2	Glucose-6-C^{14}	0.93		91.4	0.5	3.8	0.5
3	Glucose-2-C^{14}	0.96		94.0	0.4	3.7	0.5

*"Cell pool" represents material extracted from the cells by hot water.

TABLE III

THE EFFECT OF VARIOUS COMPOUNDS ON THE ISOTOPE CONTENT OF CELLULAR ASPARTIC ACID, CELLS AND MEDIUM OF *L. arabinosus* GROWN IN THE PRESENCE OF CARBON DIOXIDE-C^{14} OR GLUCOSE-C^{14}

Expt.	Labeled compound	Compound added*	Biotin level	Specific activity ratio: Aspartate / Labeled compound	Total activity ratio: Aspartate / Cells	Total activity ratio: Medium / Cells
1	$C^{14}O_2$	None	High	0.93–1.25†	0.80	0.49
5	$C^{14}O_2$	None	High	0.92–0.94	0.74	0.69
6	$C^{14}O_2$	None	Low	0.94–0.98	0.86	0.23
7	$C^{14}O_2$	0.03 *M* DL-malate	High	1.00–1.04	0.75	0.75
8	$C^{14}O_2$	0.015 *M* oxalacetate	High	0.87–0.95	0.71	0.43
9	$C^{14}O_2$	0.015 *M* oxalacetate	High	0.87–0.90		
10	$C^{14}O_2$	0.015 *M* oxalacetate	High	0.86–0.89	0.65	0.82
11	$C^{14}O_2$	0.007 *M* oxalacetate	Low	0.88–0.92	0.79	0.19
12	$C^{14}O_2$	0.001 *M* L-aspartate	High	0.144–0.145	0.74	0.22
13	$C^{14}O_2$	0.001 *M* L-aspartate	High	0.138–0.140	0.66	2.8
14	$C^{14}O_2$	0.001 *M* L-aspartate	Low	0.013	0.67	
2	Glucose-6-C^{14}	None	High	0.49	0.14	
3	Glucose-2-C^{14}	None	High	0.52	0.15	
15	Glucose-6-C^{14}	0.2 *M* pyruvate	High	0.21	0.08	

*Oxalacetic acid and sodium pyruvate solutions were sterilized by filtration but other compounds were autoclaved with the medium. In experiment 8, oxalacetic acid was added at the start of the experiment; in experiments 9 and 11, it was added at hourly intervals, and in experiment 10, at 10 to 15 minute intervals. In experiment 11, the addition was started when the medium showed faint turbidity and continued for 10 hours.

†The two values given represent the ratio calculated from the initial and the final specific activities, respectively, of the carbon dioxide.

Potter *et al.* (22) have shown that oxalacetic acid is somewhat more effective than carbon dioxide in decreasing the biotin requirement for the growth of *L. arabinosus*, and these workers came to the conclusion that biotin is involved in the fixation of carbon dioxide into oxalacetic acid, which is the precursor of aspartic acid. If this postulate were true and if oxalacetic acid were stable enough when added to growth media at 37° C. to be used as such by the growing organism, then one would expect that it would be used in preference to carbon dioxide for the synthesis of aspartic acid, and the effect would be more marked in cells grown on low-biotin medium. However in experiments 8 to 11, Table III, the organism utilized carbon dioxide-C^{14} for the synthesis of aspartic acid only slightly less when oxalacetic acid was added than when it was omitted (experiments 5 and 6). Experiments 6 and 11 were done at the same time with the same batch of medium and size of inoculum, and although there is only a 6% difference in the incorporation of carbon dioxide-C^{14} into aspartic acid, better growth was obtained when oxalacetic acid was added (yield of cells was 26 mg. dry weight in experiment 11 and 18 mg. in experiment 6). This result raises the question as to whether this growth effect of oxalacetic acid or of its decomposition products has any relation to aspartic acid synthesis. Added oxalacetic acid may well be decomposed both spontaneously and by the oxalacetic decarboxylase in the organism (10) before it has an opportunity to act as an intermediate in aspartic acid synthesis. Higher levels of oxalacetic acid were not used because of the high dilution of carbon dioxide-C^{14} that would occur from the carbon dioxide produced by decarboxylation of the compound. However, the oxalacetic acid concentrations are higher than those used by Potter *et al.* (22) and still no striking evidence was obtained for its acting as an intermediate in aspartic acid synthesis.

The author is of the opinion that experiments on the effect of oxalacetic acid on aspartic acid synthesis by growing cells of *L. arabinosus* will probably not yield conclusive evidence either as to the participation of this compound in aspartic acid synthesis or as to the role that biotin may play in the synthesis of oxalacetic acid, and that the results of such experiments will be confused by the participation of carbon dioxide from the decarboxylation of oxalacetic acid in the synthesis of aspartic acid. The presence of an aspartic-α-keto-glutaric acid transaminase in this organism (16) is better evidence for the involvement of oxalacetic acid as an intermediate in aspartic acid synthesis, but gives little information as to the role of biotin in the synthesis.

In experiment 7, Table III, the addition of malic acid to the medium did not affect the incorporation of C^{14}-carbon dioxide into aspartic acid. Malic acid is much more stable than oxalacetic acid and although the organism adaptively forms a malic decarboxylase (1), one might expect that the compound would participate in aspartic acid synthesis if it were an intermediate. Also, if malic acid were easily converted to aspartic acid, one would not expect to find that, even in the presence of malic acid, aspartic acid seems to be necessary for the synthesis of malic decarboxylase (15). Judging from available evidence, malic acid is a more unlikely intermediate in aspartic acid synthesis than is oxalacetic acid.

In contrast to the results obtained with oxalacetic acid and malic acid, the addition of aspartic acid to the medium decreases the synthesis of aspartic acid from carbon dioxide-C^{14} and the effect is much more marked with low-biotin medium (experiments 12 to 14, Table III). In experiment 12, the aspartic acid in the medium was isolated at the end of the experiment and found to contain only 43% of the radioactivity of the cellular aspartic acid, and in experiment 14, the radioactivity in the medium was too low to measure accurately. Therefore, there is not simply a constant amount of aspartic acid synthesized and a dilution of this with exogenous aspartic acid as has been found with *Escherichia coli* (24), but a net decrease in aspartic acid synthesized. This confirms the results of Lardy *et al.* (11), who obtained a similar effect of exogenous aspartic acid on the synthesis of aspartic acid by cells of *L. arabinosus*.

The synthesis of aspartic acid in *L. arabinosus* has been repeatedly reported to be concerned with biotin function. Biotin, but also substances unrelated to biotin, have been reported to have an effect on the aspartase enzyme found in some microorganisms (5, 13, 30). The present work eliminates the possibility that an aspartase enzyme which aminates fumaric acid to form aspartic acid is responsible for the synthesis of aspartic acid in *L. arabinosus*, for if such were the case, the amino group of aspartic acid would be derived entirely from the ammonia in the medium, and since fumaric acid is a symmetrical compound, the activity from radioactive carbon dioxide would be found equally in both carboxyl groups of aspartic acid and the activity from either glucose-6-C^{14} or glucose-2-C^{14} would be found equally in the α- and β-carbon atoms of aspartic acid. The experiments reported here show no

randomization of activity between the carboxyl groups or between the α- and β-carbon atoms of aspartic acid, so no detectable amount of aspartic acid synthesis occurs via fumaric acid. This differs from the results obtained by Roberts *et al.* with *E. coli*, which was found to synthesize 20 to 50% of its aspartic acid via fumaric acid (24). In contrast, the above data on the synthesis of aspartic acid by *L. arabinosus* may be explained completely by a pathway involving the β-carboxylation of pyruvic acid, or a compound related to it, followed by transamination, with no symmetrical intermediate such as fumaric acid occurring on the pathway of synthesis.

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STUDIES WITH STAPHYLOCOCCAL TOXINS

IV. THE PURIFICATION AND METALLIC REQUIREMENTS OF SPECIFIC HEMOLYSINS[1]

J. ROBINSON, F. S. THATCHER, and JEANNINE GAGNON

Abstract

A method is described whereby specific staphylococcal hemolysins may be concentrated to provide a 600-fold increase in potency as compared with the initial culture filtrate.

The progressive purification of the hemolysins was conveniently followed by the determination of the quantity of hemin (in μg.) freed from a standard suspension of erythrocytes per μg. of protein in the toxic preparation. All preparations of the electrophoretically pure hemolysins were protein in nature, but in order to effect hemolysis, preparations of similar electrophoretic mobility obtained from representatives of two different phage groups (IV and II) showed, respectively, an absolute requirement and no requirement for the presence of specific divalent cations. The initial culture filtrate was dermonecrotic and lethal to test animals. The "purified" hemolysins had neither of these properties.

Two "purified" hemolysins that showed differences in biological, electrophoretic, and chemical properties appeared to be serologically homologous. The properties of the "purified" hemolysins from either strain differed from the more generally accepted properties of alpha- and beta-hemolysins even though the culture filtrate seemed to contain these two classically recognized lysins.

Introduction

Van Heyningen's discussion (34) of the properties of four distinct staphylococcal hemolysins shows that very little is known about the nature and mode of action of these substances.

The present study describes methods of purification of specific hemolytic substances obtained from the culture filtrates of staphylococci, and some of their properties after purification to the extent of being electrophoretically homogeneous. We wish to show, also, that certain properties of the purified lysins introduced difficulties in assigning them to the classically recognized staphylococcal hemolysins, or alternatively raise some doubt as to the adequacy of existing criteria, based as they have been, on the reactions of highly impure preparations and upon sera containing antibodies against various factors in staphylococcal toxin.

The different hemolysins have been recognized chiefly by serological specificity and by their comparative ability to hemolyze erythrocytes from different animal species. Glenny and Stevens (14), Roy (29), Christie and Graydon (8), and others have established widely accepted criteria for distinguishing alpha- and beta-hemolysins of staphylococci. The alpha-hemolytic reaction was characterized by rapid lysis of sheep or rabbit erythrocytes when incubated at 37° C., but with little or no extension of lysis following further incubation at 4° C. Beta-hemolysin was considered to be unable to lyse either rabbit or sheep erythrocytes at 37° C., but caused lysis of sheep

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cells upon further incubation at 4° C. This so-called "hot–cold" reaction upon sheep erythrocytes has since been used as the main diagnostic characteristic of the beta-hemolysin.

Many deviations from this "normal" hemolytic pattern have been reported (5, 12, 20, 29, 30, 31) for which various explanations have been offered. Burnet (5) and Burnet and Freeman (7) suggested that a single antigenic substance may evoke different hemolytic reactions depending upon the extent to which it has been "toxoided" during extraction from culture media, but later investigators explain the different hemolytic behavior from staphylococcal filtrates as being due to the production of a multiplicity of different hemolysins (12, 20, 29, 31). The possibility has also been advanced that a small number of hemolysins, possibly one or two, are produced by staphylococci but that their hemolytic behavior is influenced by several other components of the culture filtrate. Some of the modifying substances referred to have included other toxic substances (8, 11, 22), metallic ions (30), enzymes (8), and other organic compounds that may be of inhibitory (24, 26, 31) or potentiative capacity (25, 27, 31) as well as unknown compounds which may be present in nutrient broth (4, 6). The extent to which each of these possibilities may be valid does not seem to have been resolved.

In order to seek clarification of some of these uncertainties, it seemed desirable to obtain preparations of distinct lytic entities separated one from the other and from the diversity of other biologically active substances present in culture filtrates of staphylococci.

Materials

Erythrocytes

Sheep blood, drawn aseptically from the jugular vein of normal animals was collected in Baxter "Transfuso Vac" bottles. Rabbit blood was drawn aseptically by heart puncture of anaesthetized animals and mixed in the proportion of three volumes of blood to one volume of a sterile solution containing 2.30 g. glucose and 1.70 g. sodium citrate in 100 ml. of solution. The erythrocytes were washed three times in either a solution containing 0.9% NaCl and 0.03% KCl or in a similar solution to which 0.03% $MgSO_4 \cdot 7H_2O$ or cobaltous acetate had been added, and they were finally suspended in the same solution at the rate of 4.0 ml. of packed cells to 100 ml. of solution.

Reagents

A source of potato starch suitable for electrophoresis was kindly provided by Hatfield Industries Limited, Hartland, N.B. Intact starch granules were separated from most of the impurities in the commercial product by the following procedure: A suspension of crude potato starch in cold distilled water was allowed to stand for 30 minutes during which time intact granules of starch and "sand" collected as a dense aggregation at the bottom of the container. Broken starch granules remained in suspension and were removed by decantation. The intact granules of starch and "sand" were separated from one another by resuspending the sedimented material in cold distilled

water. The intact granules of starch remained in suspension for 30 seconds and were separated from most of the "sand" which collected at the bottom of the vessel during this period by decantation. It was finally collected on a Buchner filter, dried by passing alcohol and acetone through the compressed cake of starch granules, and the solvents finally removed by heating for 18 hours in an oven at 50° C.

Calcium phosphate gel was prepared according to the procedure described by Keilin and Hartree (17) and tris buffer, tris(hydroxymethyl)aminomethane, was obtained from the Sigma Chemical Company.

Methods

Methods applied in the past to the partial purification of staphylococcal hemolysins have included: (*a*) precipitation from the culture filtrate by the respective use of acetic acid to pH 5.0 (7), ammonium sulphate (15), trichloracetic acid, and acetone at pH 4.0 for alpha-hemolysin and borate buffer and acetone at pH 9.0 for beta-hemolysin (10, 13); acetic acid and methanol (16, 35); 40% ethanol at −20° C. (33); (*b*) paper chromatography (21), a method which did not allow elution of the proteins from the paper; (*c*) zone electrophoresis using filter paper and starch, respectively, as the supporting media (2, 33).

For present purposes, ethanol at −20° C. and ammonium sulphate were used as precipitants; calcium phosphate gel was used as an adsorbent in accord with the method of Keilin and Hartree (17), who found the gel useful in the purification of proteins. These steps were followed by a final separation involving the use of zone electrophoresis. A number of modifications of the electrophoretic method were applied using glass-fiber filter paper and conventional cellulose filter paper (Schleicher and Schuell 598) as the supporting media (3, 36) as well as starch after the method of Kunkel and Slater (18). The use of starch, in our experience, offered advantages in reproducibility, ease of recovery of protein fractions, the degree of separation, and in the allowance of a larger amount of specimen to be subjected to separation in a single test. Results from electrophoretic studies will be confined in this report to those dependent on the use of starch. Descriptive detail is presented below.

Staphylococcus Cultures and the Production of Crude Hemolysins

The cultures used for this study were typical coagulase-positive representatives of *Staphylococcus aureus*.* One, L.16, was involved in a food-poisoning incident and some of its properties have already been described (32). The second, strain 92, was isolated from a hospitalized patient with a pyogenic infection. Strain L.16 is a member of phage group IV; 92 was later found to be a member of phage group II.

*In the sense of Bergey's Manual of Determinative Bacteriology, 7th edition. These same cultures have been described previously as *Micrococcus pyogenes* var. *aureus*.

The organisms were grown in Petri dishes on the semisolid medium developed by Dolman and Wilson (9), and incubated for 3 days at 37° C. in an atmosphere of 10% CO_2 and 90% O_2. The bacterial cells and much of the agar were removed from an aggregate of an appropriate number of such cultures by centrifuging at 5000 r.p.m. A crude concentrate of hemolysins and other proteins present in the supernatant was obtained by precipitation using ethanol at −20° C. in accord with the method previously described (33). The precipitate was recovered by centrifuging at −10° C., dissolved in a minimum volume of distilled water (10–20 ml.) at room temperature, and dialyzed 18 hours against 0.0065 *M* phosphate buffer, pH 7.0, at 4° C. The resultant slight precipitate was removed by centrifugation and the supernatant solution lyophilized. The residual solid was used as a crude source of hemolysins for further experimentation. Preparations made in this manner will be referred to hereon as the "crude source".

Electrophoretic Procedure

The number of different protein fractions in the "crude source" and also after further refinement was determined by zone electrophoresis after the method of Kunkel and Slater (18) using starch as the inert supporting medium and 0.01 *M* tris buffer at pH 7.3 as the continuous phase with an applied potential of 350 volts. The electrophoretic separation was continued for 18 hours at room temperature. The starch "column" was held in a trough of dimensions 80 cm. in length, 15 mm. in width, and 8 mm. in depth. It was constructed of the plastic material "Perspex". The eventual distribution of proteins in the starch column was estimated by dividing it into segments 1.5 cm. in length, and extracting each segment with 5.0 ml. of tris buffer at the same pH as that used during electrophoresis. A quantitative determination of protein was made from 0.5-ml. portions of each of the extracts by the colorimetric method of Lowry *et al.* (19). The resultant values expressed in μg. were then related to the capacity of each extract to hemolyze erythrocytes of sheep and of rabbit at 37° C. and also after the "hot–cold" treatment. The ability of many of the extracts to effect emesis in cats, dermonecrosis in rabbits, and death of rabbits or cats was also determined.

Biological Tests

Emesis in cats was determined after intraperitoneal injection in accord with the method of Matheson and Thatcher (23). Dermonecrosis was determined by intradermal injection of rabbits and lethality by injection of graded levels of specific fractions into the lateral ear vein of rabbits or as a fatal response to the injection of cats following a test for emesis.

Determination of Hemolysis

Hemolysis was estimated by determining the amount of hemin freed from preparations of washed erythrocytes of rabbit and of sheep after exposure to test preparations for appropriate periods at 37° C. and again after a further incubation at 4° C. Equal volumes of the suspension of washed erythrocytes

and various dilutions of test hemolysins were mixed after temperature equilibration, incubated for specific periods, and residual intact erythrocytes removed by centrifuging. The concentration of hemin present in the supernatant solution was determined colorimetrically by the method of Rimington (28), which is dependent upon estimation of a pyridine hemochromogen by its absorption at 550 mμ. A Bausch and Lomb "Spectronic 20" colorimeter was used for this purpose.

Fractionation Procedure

Preliminary study showed that calcium phosphate gel prepared by the method of Keilin and Hartree (17) adsorbed hemolytic factors. Also, the hemolysins were removed from a solution of the "crude source" by 66% saturated ammonium sulphate.

Accordingly, calcium phosphate gel was used to adsorb the hemolytic agent from a solution of the "crude source". Much of the pigment and some extraneous proteins were removed by elution of the gel with 0.1 *M* phosphate buffer at pH 7.3 after which the hemolysins were eluted by treatment of the gel with 1.0 *M* phosphate buffer. The resulting solution was dialyzed overnight against cold running tap water, the small amount of precipitate which developed was removed by centrifugation, and the supernatant solution was lyophilized. To the lyophilized material reconstituted in a minimum volume of water, a saturated solution of ammonium sulphate at pH 7.0 was added to a final concentration of 66% saturation. The precipitate which formed was collected by centrifugation, dissolved in a minimum volume of water, and dialyzed overnight against running tap water at 12° C. The precipitate which formed during dialysis and the supernatant were each tested for hemolytic activity both before and after being subjected to starch electrophoresis.

Serological Tests

Fractions shown to possess hemolytic activity were titrated serologically against commercial staphylococcus antiserum having a high anti-alpha titer (Connaught Laboratories) and against an anti-beta preparation partially "purified" by specific absorption and kindly provided by Dr. C. E. Dolman of the University of British Columbia. Electrophoretically purified hemolysins were used independently as antigens in the preparation of antisera from rabbits. Serological specificity was determined also by the "strip-plate" method of Elek and Levy (11).

Spectrophotometric Determinations

The infrared and ultraviolet spectra of specific hemolytic specimens were determined with the respective use of the Perkin Elmer double-beam recording infrared spectrophotometer and the Beckman spectrophotometer.

Results

Preliminary experiments were made to determine the extent to which hemolysin activity was related to time and to hemolysin concentration. The results are presented in Figs. 1 and 2. On the basis of these preliminary

findings, the activity of all hemolysin preparations was determined during a 20-minute incubation period at 37° C. and the results expressed as μg. of hemin in 1.0 ml. of supernatant solution freed by a quantity of hemolysin containing 1.0 μg. of protein. Other preliminary data showed that maximum hemolytic activity could be obtained at pH 7.0 to 7.5. Subsequent tests, therefore, were conducted in the presence of 0.01 *M* buffer at pH 7.4 containing 0.9% NaCl and 0.036% KCl. Additions to this basal solution, usually 0.03% $MgSO_4.7H_2O$ or 0.03% cobaltous acetate, were made as required.

The initial culture filtrate caused the following reactions: (*a*) hemolysis of sheep and rabbit erythrocytes at 37° C., (*b*) intensified hemolysis of sheep and rabbit erythrocytes when further incubated at 4° C., (*c*) emesis in cats, (*d*) dermonecrosis in rabbits, and (*e*) death of cats and rabbits. The growing

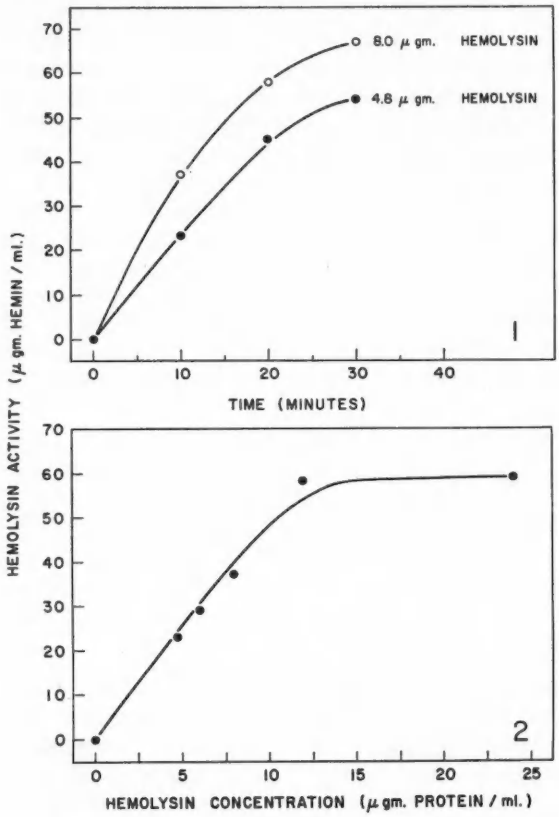

FIG. 1. Hemolysis of sheep erythrocytes by hemolysin from alcohol precipitate of culture filtrate of L.16. Toxin dissolved and erythrocytes suspended in basal salts solution containing 0.05 *M* $MgSO_4.7H_2O$.

FIG. 2. Hemolysis of sheep erythrocytes by graded levels of hemolysin from alcohol precipitate of culture filtrate of L.16.

culture on blood agar and the filtrates obtained from Dolman's medium caused lysis characteristic of alpha- and beta-hemolysins, each of which was inhibited by commercial antitoxin as shown by the "strip-plate" method of Elek and Levy (11).

The electrophoretic separation of a specimen of the "crude source" from culture L.16 into five protein components of differing mobility is illustrated in Fig. 3. This figure expresses the concentration of protein at progressive distances from the origin in a starch "column" subjected to 350 volts at room temperature for 18 hours using 150 mg. of specimen suspended in 0.01 *M* tris buffer at pH 7.3. The five "peaks" of protein content are numbered from 1 to 5 in order of mobility towards the cathode.

It will be noted that one fraction remained at the origin. This protein (protein No. 1) had no demonstrable activity as a hemolytic, dermonecrotic, lethal, or emetic toxin. This was true also for the most mobile of the proteins (No. 5). Protein No. 2 was not hemolytic, moved at 0.3 cm./hr., and was shown to be emetic but not lethal to cats, a dual property demonstrated by no other test preparation. Protein No. 3 moved at 1.0 cm./hr., failed to hemolyze sheep erythrocytes at 37° C., but caused extensive hemolysis upon further incubation for 1 hour at 4° C. Protein No. 4 migrated at 1.5 cm./hr. and hemolyzed sheep erythrocytes at 37° C. Neither of the actively hemolytic fractions were dermonecrotic but they were lethal to approximately 50% of the cats injected. Rabbits injected with 1 ml. of a culture filtrate containing a lysin titer (sheep) of 1:80 were killed in less than 30 minutes. Specimens

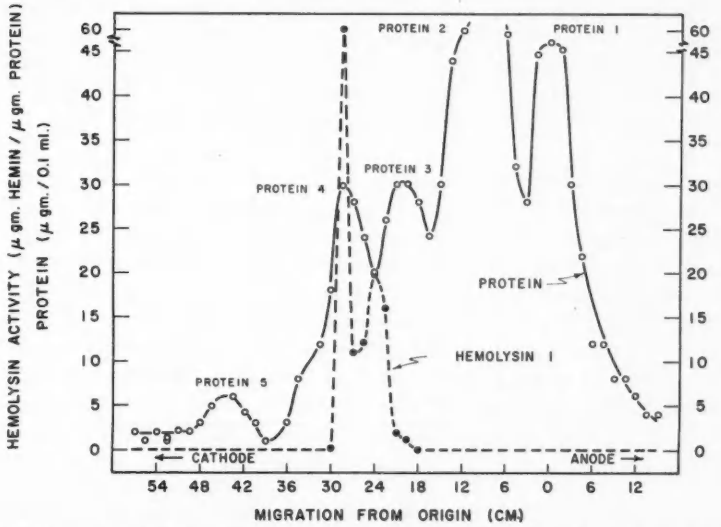

FIG. 3. Separation of the protein constituents of the alcohol precipitate of a culture filtrate of L.16, using a starch electrophoresis procedure with 0.01 *M* tris buffer, pH 7.4. Hemolytic activity evaluated using sheep erythrocytes suspended in basal salts solution containing 0.05 *M* $MgSO_4.7H_2O$.

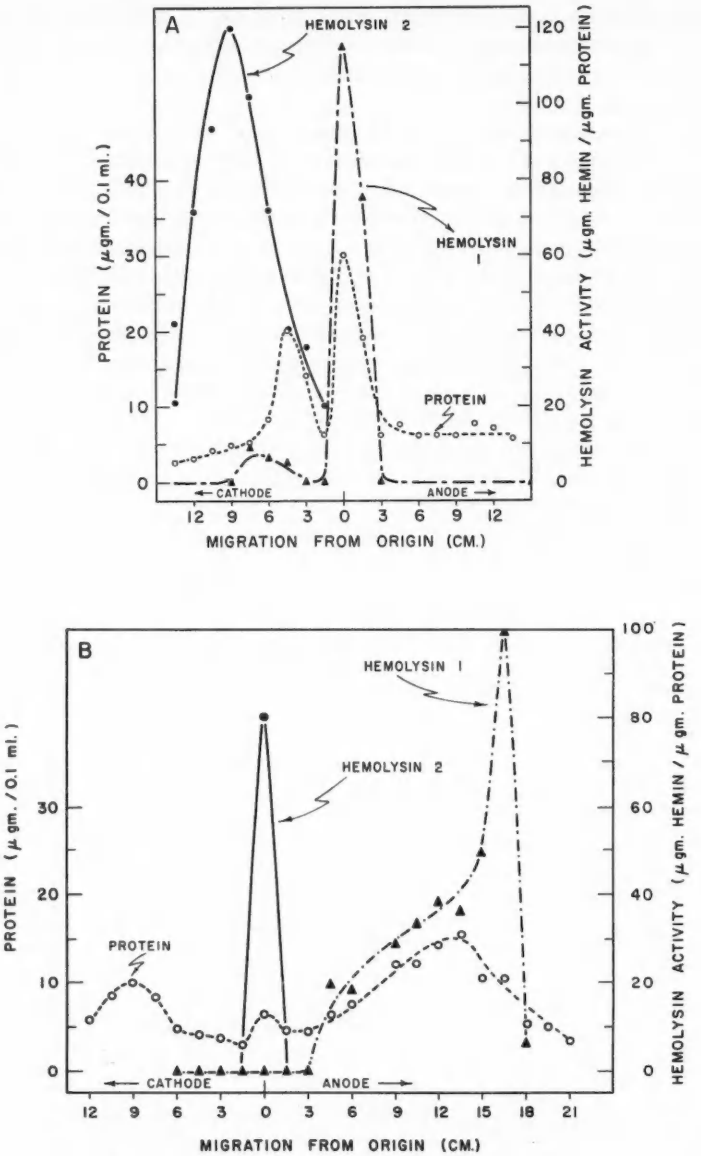

FIG. 4. Separation by means of the starch electrophoresis procedure of the protein constituents in 45 mg. of material eluted from calcium phosphate gel. Hemolysin activity evaluated using sheep erythrocytes suspended in basal salts solution containing 0.05 *M* $MgSO_4.7H_2O$.

A. With 0.01 *M* acetate buffer, pH 5.0.

B. With 0.01 *M* tris buffer, pH 7.4.

at lower degrees of dilution killed the test rabbits in a few seconds. The minimum amount of either of the purified lysins to cause death of rabbits (in 2 hours) had a hemolytic titer of 1:4000. Rabbits receiving comparable injections at 1:3000 titer survived.

Electrophoresis at pH 5.0 and at pH 7.4 of the eluate obtained after adsorption of a specimen of crude source on calcium phosphate gel revealed only two protein "peaks" as shown in Figs. 4A and 4B. In each instance, both proteins were hemolytic. Of the two hemolytic fractions isolated at pH 5.0 one remained at the origin and the other migrated toward the cathode at 0.6 cm./hr. The former hemolyzed sheep erythrocytes after incubation at 37° C. and to an increased degree after further incubation for 1 hour at 4° C. These two fractions, initially, were designated tentatively as alpha-hemolysin and beta-hemolysin according to the classification suggested by Glenny and Stevens (14) but because of subsequent uncertainty of this designation have been referred to in Figs. 4A and 4B as hemolysin 1 and hemolysin 2. Similarly, at pH 7.3, two fractions with hemolytic activity were isolated. A fraction with hemolytic activity suggestive of the beta-lysin remained at the origin and one with alpha-type hemolytic activity migrated towards the anode at approximately 0.8 cm./hr. These results suggest, therefore, that the isoelectric point of hemolysin 1 is approximately pH 5.0 and that of hemolysin 2 is approximately pH 7.3. It will be noticed that at pH 5.0, maximum hemolysis caused by hemolysin 1 was coincident with the maximum concentration of protein. With respect to hemolysin 2, maximum hemolysis at this pH was not so coincident, but instead, corresponded to a point of protein concentration about half that of the maximum. At pH 7.3, however, these two observations were reversed; namely, maximum hemolysis by hemolysin 2 was coincident with the maximum concentration of protein, but the two maximal values of hemolysis and protein concentration from hemolysin 1 were no longer coincident.

Recovery of two fractions of mobility corresponding to that of the specimens derived from the calcium phosphate gel was also provided by electrophoresis of specimens obtained by use of 66% saturated ammonium sulphate. These last two treatments thus separate hemolysins from the non-hemolytic emetic fraction (enterotoxin) and from the two other non-hemolytic proteins from the crude source. When fractionation was accomplished by progressive application of the three procedures, namely, precipitation with alcohol at −20°C., adsorption by calcium phosphate gel, and precipitation by ammonium sulphate, the same two peaks of hemolytically active proteins were obtained and with increased activity of the final fractions as compared with the use of the gel or of the ammonium sulphate alone. (This is shown among the results presented in Table III.)

Preparations of the two final fractions each having a hemolytic titer of 1:10 were shown to be completely inhibited by additions of dilutions (1:1024 and 1:512) of commercial staphylococcal antitoxin containing 10,000 units/ml. and *also* by Dr. Dolman's preparation of beta-antitoxin. They were each

inhibited by their homologous antisera which, however, could not be shown to be serologically distinct; that is, each antitoxic serum inactivated either antigen to the same degree. These results are expressed in Table I.

The influence of chelating agents and of specific cations upon the hemolytic activity is shown by the data in Fig. 5. Ethylenediaminetetraacetic acid (EDTA) and citrate completely inhibited hemolysis of sheep erythrocytes whether at 37° C. or after the "hot–cold" treatment. The limiting concentration to provide complete inhibition for each reagent was, respectively, 7.5×10^{-5} *M* and 3.0×10^{-3} *M*. Addition of magnesium sulphate reactivated the hemolysin. Presumably, citrate and EDTA inhibit hemolysis of sheep erythrocytes by virtue of the capacity of these compounds to chelate metallic ions essential for the hemolytic reaction. In addition to magnesium, the salts of cobalt, nickel, iron (ferrous), and manganese reactivated the hemolysin

TABLE I

NEUTRALIZATION OF HEMOLYSINS ISOLATED FROM CULTURE L.16 USING VARIOUS ANTISERA

(Results expressed as the reciprocal of the highest dilution of antiserum which prevents hemolysis of sheep erythrocytes)

Source of antiserum	Hemolytic preparations		
	Hemolysin 1	Hemolysin 2	Hemolysin in crude source
Staphylococcus antitoxin (Connaught, 10,000 units)	1024	512	512
Anti-beta hemolysins (Dolman)	256	128	128
Antihemolysin 1	256	256	—
Antihemolysin 2	128	128	—
Normal serum	4	4	—

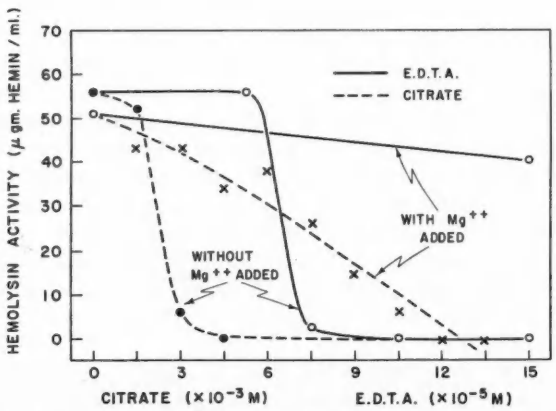

FIG. 5. Effect of citrate and ethylenediaminetetraacetic acid, pH 7.0, on hemolysis of sheep erythrocytes in the presence and absence of added Mg^{++}. Alcohol precipitate of culture filtrate of L.16 used as a source of hemolysin.

which had been inhibited by EDTA. The salts of sodium, potassium, calcium, and zinc were without effect in this capacity. These results, which are illustrated in Table II, suggest that the hemolysins might be resolvable into a protein moiety (apohemolysin) and a metallic cofactor.

TABLE II

COMPARATIVE EFFECT OF METALLIC IONS ON THE ACTIVITY OF HEMOLYSIN PREVIOUSLY INHIBITED BY EDTA

Ionic additions (50×10^{-5} *M*)	Hemolysin activity (μg. hemin/μg. protein)
Co^{++}	40.0
Ni^{++}	24.5
Fe^{++}	20.0
Mg^{++}	18.7
Mn^{++}	20.0
Ca^{++}	Nil
Zn^{++}	Nil
No additions	Nil

Using only salts of sodium or potassium in the medium in which erythrocytes were suspended, the effect of progressive purification and of added salts of magnesium upon hemolysis are expressed by the data in Table III. A culture filtrate from strain L.16 vigorously hemolyzed erythrocytes of sheep and of rabbit. In the absence of added magnesium, hemolysis of erythrocytes of both animals was reduced after the cold-ethanol treatment and still further curtailed after elution from calcium phosphate gel or precipitation with ammonium sulphate. The proteins recovered after electrophoresis demonstrated no hemolysis under the conditions of these tests. However, hemolysis of sheep cells at 37° C. and also after "hot–cold" treatment was restored upon addition of magnesium sulphate. Additions of cobaltous acetate exerted

TABLE III

EFFECT OF MAGNESIUM (25×10^{-5} *M*) ON HEMOLYTIC ACTIVITY OF PREPARATIONS FROM CULTURE FILTRATES OF L.16 AT VARIOUS STAGES OF PURIFICATION USING ERYTHROCYTES FROM RABBIT AND SHEEP

(Activity expressed as μg. of hemin liberated by hemolysin containing 1.0 μg. protein)

Fraction	Source of erythrocytes			
	Rabbit		Sheep	
	Mg added	No Mg	Mg added	No Mg
Culture filtrate	1.4	1.2	1.0	1.0
Alcohol precipitate	1.7	1.0	15.0	4.2
Calcium phosphate eluate	Nil	Nil	30.0	7.2
$(NH_4)_2SO_4$ precipitate				
A. Water soluble	Nil	Nil	69.6	Nil
B. Water insoluble	Nil	Nil	280.0	Nil
Starch electrophoresis of B	Nil	Nil	670.0	Nil

a similar effect. These salts were without effect on the recovery of hemolytic action against rabbit cells. A 650-fold concentration of hemolysin was obtained during the purification process outlined in Table III.

The smallest amount of these two salts that would fully reactivate hemolytic action of the two hemolytic proteins obtained after electrophoresis is shown by the data expressed by Fig. 6. Hemolysin 1 (hemolysis of sheep erythrocytes at 37°) was activated by 1×10^{-5} *M* $MgSO_4$ or 0.75×10^{-5} *M* cobaltous acetate. The minimum amounts of these salts to reactivate hemolysin 2 ("hot–cold" hemolysis) were 2.5×10^{-4} *M*.

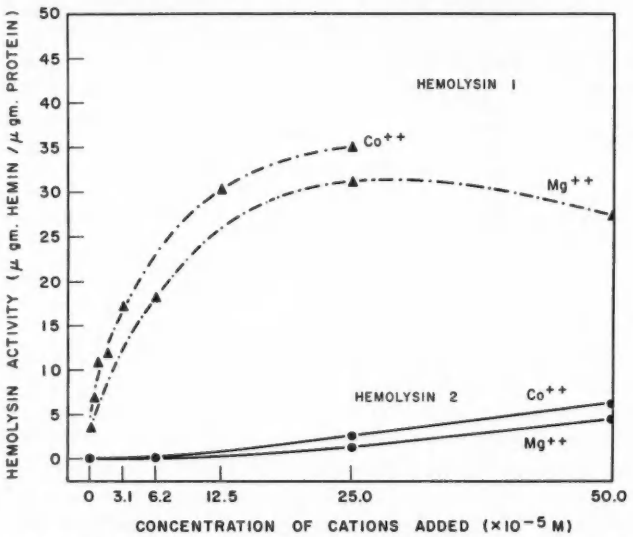

FIG. 6. Effect of cobalt and magnesium on the reactivation of hemolysins 1 and 2 separated by the starch electrophoresis procedure.

The infrared and the ultraviolet spectra of the two hemolysins recovered by starch electrophoresis are shown respectively in Figs. 7 and 8. It will be noted that the two hemolysins even though of differing mobility and with differing biological properties have closely similar infrared spectra, but their ultraviolet spectra are separable by the presence in the spectrum of hemolysin 1 of a distinct "peak" of absorption at 260 mμ. This difference, according to results presented by Ajl *et al.* (1), suggests the presence of a small amount of nucleoprotein in hemolysin 1 but not in hemolysin 2. The foregoing results collectively indicate that the two isolated hemolysins are closely similar proteins each having a requirement for specific metallic divalent cations.

The foregoing results are based on hemolysins produced by strain L.16, a member of the phage group IV. Preliminary experiments with strain 92 (phage group II) showed that this culture produced two hemolysins recoverable by the starch electrophoresis procedure. One hemolysin lysed rabbit

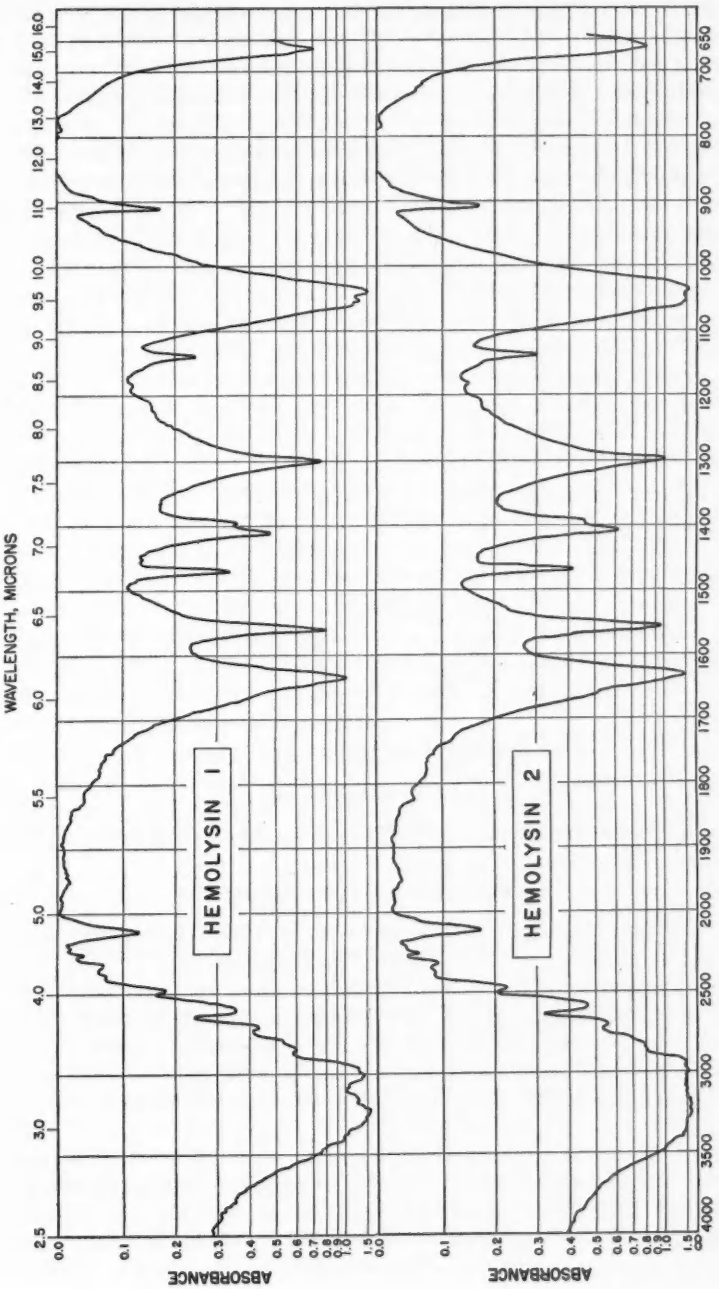

FIG. 7. Infrared spectrum of hemolysins 1 and 2. KBr disks contained 1% of each hemolysin.

erythrocytes at 37° C. and was also able to lyse both rabbit and sheep erythrocytes under conditions of the "hot–cold" test; the second hemolysin had no hemolytic effect upon cells of rabbit or sheep at 37° C., but hemolyzed cells of *rabbit only* by the "hot–cold" treatment. Neither hemolysin was lethal to rabbits. Neither of these hemolysins revealed a requirement for magnesium, manganese, or cobalt. These preliminary data suggest that different hemolytic entities may be produced by different staphylococcus strains, an observation which will be extended by comparable examination of strains from other phage groups.

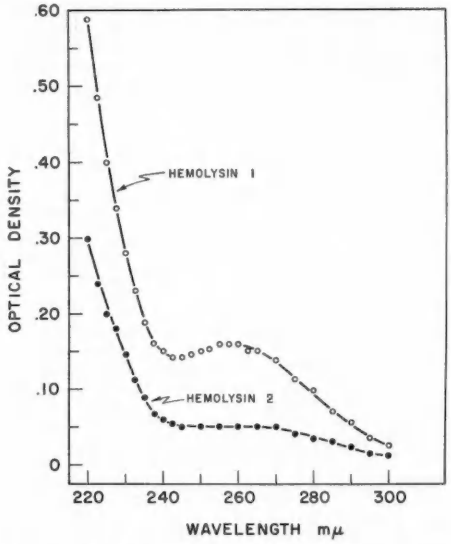

FIG. 8. Ultraviolet spectrum of hemolysins 1 and 2. Hemolysins prepared as 0.08% solutions in water.

Discussion and Conclusions

The colorimetric method of determination of hemolysis described herein and which is based on the quantitative determination of a pyridine hemochromogen prepared from the hemin liberated from lysed cells is a useful method for determining progressive purification of hemolysins. Where large numbers of hemolytic determinations are required it offers advantages in the saving of time, and in accuracy and reproducibility in comparison with the time-honored but laborious tube-dilution method, with its often uncertain end point.

A several-hundred-fold purification of specific hemolytic proteins produced by staphylococci has been attained by the progressive use of precipitation by 40% ethanol at −20° C., adsorption, and elution from calcium phosphate gel; precipitation by 66% saturated ammonium sulphate and zone electrophoresis using potato starch as the inert carrier.

Two hemolytic proteins were recovered in this way from strain L.16 (phage group IV) of *S. aureus* each of which demonstrated a positive requirement for the presence of metallic cations in order to effect hemolysis. These hemolysins differ in metallic requirement from two proteins of similar electrophoretic mobility produced by strain 92, a member of phage group II and newly isolated from a pyogenic infection. For the lysins of this strain, no metallic requirement could be demonstrated. The purified lysins of either strain did not possess the property of dermonecrosis.

The authors are unable to identify with certainty these hemolysins with the "classical" alpha- or beta-staphylococcal lysins even though they have several properties in common with them. The hemolytic proteins recovered from strain L.16 are completely inactivated by their homologous antisera. They are also inactivated by commercial antitoxin containing a high anti-alpha titer. This might suggest that they were homologous with the alpha-lysin used as antigen in the preparation of the commercial product. But one of the proteins while able to lyse sheep cells at 37° C., did not lyse rabbit cells; whereas the other lysed sheep cells *only* after a "hot–cold" reaction. The classical alpha-lysin is considered to lyse rabbit cells at 37° C., the beta-lysin is typified by the "hot–cold" reaction with sheep cells. Further apparent discrepancy, however, is revealed by the fact that each purified hemolysin was inactivated by a preparation absorbed to be specific for beta-lysin (kindly given us by Dr. C. E. Dolman some three years ago).

The hemolytic proteins obtained from strain 92, while of similar electrophoretic mobility to those from strain L.16 differ markedly from the latter in not requiring metallic cations. Neither of these appear to be identical with the classical alpha- or beta-lysins.

For hemolysins 1 and 2, differing pH values of a suspending medium modified the degree of coincidence of hemolytic activity and of protein concentration provided by electrophoretic distribution. This may suggest that in each instance either the pH change modified the hemolytic activity of the protein so that only part of it functioned hemolytically and that this part had a slightly changed mobility, or else each "peak" of protein was a complex of several entities of very similar properties of partition and mobility among which a small degree of relative change in mobility was induced at the experimental pH values used.

Even though this latter observation may have been true, our "purified" preparations nevertheless possess collective properties more restricted than those generally associated with the classical lysins. This may be due to partial inactivation of the original hemolytic molecule (or molecular aggregate), or else the classical concept is in part an artifact resulting from the use of highly impure preparations and of impure antisera. This latter point of view may be in accordance with the suggestion by Elek and Levy (11). It is quite possible that the multiple effects ascribed to the classical lysins may be the sum total of several entities acting additively, synergistically, or in the nature of an apoenzyme–coenzyme complex.

Such a conclusion when related also to the possession of differing hemolytic properties by the hemolysins of different strains may well be contributory to the lack of unanimity in the literature. Efforts to seek the factors responsible for lethality, dermonecrosis, and other possible biological reactions are planned.

It is of interest to note an apparent serological homogeneity between two proteins of demonstrated differences in biological, electrophoretic, and chemical properties.

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SOIL EXTRACT IN SOIL MICROBIOLOGY[1]

NORMAN JAMES

Abstract

Soil extract contains unidentified growth factors not present in other materials. In this study the extract was considered critically in so far as various treatments affect numbers of bacteria developing on soil-extract agar. Dilution of the extract to 1/10th concentration produced lower counts in most soils. Calcium sulphate and calcium carbonate, used as precipitants, failed to produce more or clearer extracts. Unheated extract provided the nutritional requirements for a considerable fraction of the bacteria in soil, but not for as large a fraction as did heated extract. Dried extract produced lower counts than did freshly prepared extract. The addition of 0.02% dipotassium acid phosphate to soil extract increased counts significantly. The use of the proportion of soil and water that gave the amount of extract desired, without the addition of water after filtration, produced the maximum amount of extractives from the amount of soil used.

Introduction

From the beginning of the study of soil microbiology, water extracts of soil have been used to obtain counts of bacteria in soil, both as the main source of nutrients and as an additive in media containing known organic materials and mineral salts. The practice was based on the belief that soil, which supports large populations of microorganisms, contains the nutritional substances essential for their growth—an assumption that was readily verified. Not only did soil-extract agar prove acceptable for culturing a significant fraction of the soil population, but it was found to be superior to certain defined media (Waksman (9), Smith and Worden (7), and Taylor (8)). Soil-extract agar gave higher and less varying replicate counts, and on it there was relative freedom from spreader colonies of bacteria and from fungi. Undoubtedly, this superiority would have been more pronounced if in all cases comparisons had been made after incubation for 14 days. Many colonies on soil-extract agar are small and appear only after prolonged incubation. Recent evidence has established that none of the media tested by these investigators could equal soil-extract agar. Lochhead and Chase (4) reported that 19% of 332 cultures isolated from soil on soil-extract agar would not grow in a basal salts medium to which were added glucose, 10 amino acids, and 7 growth factors, or in a medium containing the same salts, glucose, and yeast extract. Some years later Lochhead and Thexton (5) found that 20 of 75 cultures requiring soil extract in the basal salts, glucose, yeast extract medium grew equally well in the same medium with soil extract replaced by vitamin B_{12}. The soil extract used had a vitamin B_{12} content of 1.96 mμg./-ml., a portion of which could have been synthesized by soil bacteria. Twenty strains of bacteria from soil cultured in a medium lacking vitamin B_{12} produced in the filtrates vitamin B_{12} in amounts ranging from 152.5 to 0.10 mμg./ml.

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Contribution from the Department of Microbiology, the University of Manitoba, Winnipeg, Manitoba.

When a filtrate of each of the 12 strains that provided 0.08 mμg./ml. or more of the vitamin in the test medium was added to the test medium, growth of 30 test organisms, selected because of their vitamin B_{12} requirement, was practically equivalent to that obtained in the check medium, which contained vitamin B_{12}. There remained 55 or approximately 10% of the original 534 cultures isolated for which some factor, or factors, present in soil extract and not in yeast extract and different from vitamin B_{12}, was essential for growth. For one species in a group that represents a significant portion of the indigenous soil flora this factor has been studied in detail (6) and found to be different from any known growth factor. It is synthesized in a medium containing only inorganic salts and sugar by *Arthrobacter pascens* and was found to be nutritionally equivalent to soil extract for a closely related organism recently named *Arthrobacter terregens*, and essential for its growth.

Many investigators have discounted the value of soil extract because of certain inherent weaknesses attributed to it. Three of these objections appear worthy of brief consideration. (*a*) Its composition is unknown—an objection likewise applicable to other ingredients (meat extract, yeast extract, and serum) in accepted use in media. The fact that its composition is unknown provided the stimulant that led to the important contributions concerning growth factors in soil extract referred to above and unquestionably yet to be made. (*b*) Its composition probably varies, a probability which holds true of other extracts. The problem of variation in composition in so far as it affects counts of bacteria on soil-extract agar has been dealt with by Smith and Worden (7). Ten soils with greater differences than would be found in field soils normally used for preparing extracts were used to make 10 soil-extract agars. These agars were used to obtain counts on five representative samples of soil. For each sample the differences among counts on the 10 media were not greater than would have been expected from 10 replicate platings on one of the media. The problem of variation in composition in relation to growth factors has been considered briefly by Lochhead and Chase (4). An extract prepared from a fertile soil added to a basal salts, glucose, yeast extract medium produced good growth of 15 test cultures known to require soil extract for growth, whereas an extract prepared from a poor soil produced good growth in only two of the cultures, and moderate growth in two others. This variability in composition could be minimized by using in all cases soil from fertile fields, as would represent a normal procedure. (*c*) The preparation of soil extract is cumbersome, particularly with a soil of high moisture-holding capacity. This is a practical objection. It seems reasonable to conclude that, in the light of present knowledge, none of these prejudices has little significance.

Because of the recognized superiority of soil-extract agar in obtaining counts by the plating method and of the recent interest in soil extract as the source of growth factors, and notwithstanding the prejudices referred to, the present study was undertaken. It represents a critical consideration of

various treatments of soil extract and of its use in the preparation of soil-extract agar. The extent of variation in procedures for preparing soil-extract agar is indicated in Table I.

TABLE I

PREPARATION OF SOIL-EXTRACT AGAR IN DIFFERENT LABORATORIES

	Fischer (1909)	Löhnis (1913)	Smith and Worden (1925)	Fred and Waksman (1928)	Lochhead and associates (1938)
Soil, g.	1000	1000	500	1000	1000
Water, ml.	1000	800	1200	1000	1000
Precipitant	Na_2CO_3 (1 g.)	—	—	A little $CaCO_3$	A little $CaSO_4$
Filtrate increased to	1000	800	1000	1000	1000
K_2HPO_4, g.	2.0	—	0.5	0.5	0.2
Agar, g.	12	15	20	12.5	15

Materials and Methods

The soil used to prepare the extracts was a clay of the Red River Association (1) obtained from a freshly ploughed field that had been under cultivation for approximately 40 years. About 200 lb. were held in a loosely covered container in the laboratory and portions were used as required over a period of 8 months. When first used it had 23% moisture and 89% moisture-holding capacity, each based on oven-dried weight. The extracts were prepared as follows: Heat 1 kg. soil in 1 liter tap water at 121° C. for 30 minutes. Add 0.5 g. calcium sulphate and mix. Filter and increase the filtrate to 1 liter using tap water. The soil-extract agar was prepared by adding 0.2 g. dipotassium acid phosphate and 15 g. agar to 1 liter extract, heating to dissolve, cooling, adjusting to pH 6.8, and heat-sterilizing. This medium was used to obtain counts on different soils as defined in the studies that follow. It was used along with soil-extract agar differing in the manner indicated in each study. Twenty replicate plates were prepared from one dilution of the sample under study, the standard medium being added to 10 plates and the other medium to the remaining 10 plates. Incubation was at 26° C. for 14 days. In a preliminary trial one set of 10 replicate plates from one dilution produced a mean count of 25 at 7 days, and of 34 when recounted at 14 days. Additional colonies were present on these plates when re-examined at 28 days. However, incubation for so long would introduce complicating problems. The use of a single dilution eliminated differences in count attributable to factors (3) other than medium and gave counts on the two media that were comparable but had no valid relationship to those from another sample. Each recorded mean was calculated from 10 counts, which varied according to expectancy on the basis of random sampling from one dilution (2).

Experimental

Concentration of Soil Extract in Soil-Extract Agar

Two concentrations of soil extract were compared: the first prepared as outlined under methods, and the second, 100 ml. extract diluted in 900 ml. tap water (10% concentration). Ten samples representing eight soils were used. The soils are defined and the results presented in Table II. One

TABLE II

EFFECT OF CONCENTRATION OF SOIL EXTRACT IN SOIL-EXTRACT AGAR ON NUMBERS OF BACTERIA. TWO MEAN COUNTS, EACH FROM 10 REPLICATE PLATES FROM ONE AND THE SAME DILUTION OF SOIL

Soil*	Full strength	10%
1*a*	47	42
1*b*	83	58
1*c*	125	96
2	73	67
3	21	22
4	143	120
5	172	157
6	90	73
7	56	55
8	64	64

*1: Clay, cultivated, Red River Association (1).
2: Silty clay loam, pastured, Lakeland Association.
3: Loam, virgin, Garson Association.
4: Green house potting soil.
5: Same as 4, washed from wheat seedling roots.
6: Coarse sandy clay, cultivated, Birds Hill Association.
7: Sandy loam, cultivated, St. Cloud, Minnesota.
8: Silty clay loam, saline phase, Lakeland Association.
a, *b*, and *c*: Different samples from one soil type.

point is obvious. The full-strength extract gave higher counts in most of the trials. A second observation has to do with size of colony. In general, colonies were larger on full-strength extract agar, and counting was less tedious. This finding appears to justify the belief that differences in concentration of extracts prepared by different investigators had little effect on numbers of colonies developing on the media. Differences would be much smaller than the 1.0 to 0.1 difference used in this study.

Precipitants in the Preparation of Soil Extract

Three soil extracts were prepared simultaneously from three 1000 g. aliquots of one soil, each in 1000 ml. water. After heating at 121° C. for 30 minutes aliquot (*a*) was filtered at once, aliquot (*b*) was treated with 0.5 g. calcium sulphate for 5 minutes and then filtered, and aliquot (*c*) with 0.5 g. calcium carbonate for 5 minutes and then filtered. Observations on time to filter, clearness of filtrate, amount of filtrate, and pH of filtrate after being increased to 1 liter by the addition of water and being sterilized, were recorded. The experiment was repeated with two other soils. With each soil the use of either precipitant failed to produce any difference other than a slight change in pH of the filtrate from two of the soils (from pH 7.8 to 8.0 in one case and from

pH 8.5 to 8.9 in the other) calcium carbonate producing a greater change than calcium sulphate in each case. It should be noted that the soils used produced filtrates in the alkaline range, and that acid soils might respond to these precipitants in a different way.

Heated Versus Unheated Soil Extract

A simple experiment was carried out to determine the effect of heat in preparing extract and sterilizing it on the number of bacteria developing on soil-extract agar. Two extracts were prepared from aliquots of one soil, each with double the concentration of extractives: the extract from 1 kg. soil was increased to 500 ml. (not to 1000 ml.). The first aliquot was heated to extract the solutes, filtered, and water was added to raise the volume to 500 ml. This extract was heat-sterilized. The second extract was prepared by soaking the other 1000 g. aliquot in 1000 ml. water at 45° C. for 3 days, adding water to compensate for evaporation, filtering, and adding water to raise the volume to 500 ml. This extract was Seitz-filter sterilized. The two extracts were used to prepare three soil-extract agars: one with heated extract, one with filtered extract, and one with half the quantity of each extract. The other portion of each medium, consisting of 500 ml. water, double concentration of dipotassium acid phosphate and of agar, and 0.2 ml. 1/10th normal hydrochloric acid,* was sterilized by heat, cooled to 50° C., and mixed with the extract, likewise at 50° C., immediately before the plating. The three media were used for counts of bacteria in five soils. The soils are defined and the results presented in Table III. Two points are apparent. The unheated extract provided the nutritional requirements for a significant portion of the population in each soil, but not for as large a portion as did the heated extract. The mixing of the two extracts failed to produce larger counts than did either of the extracts. Whether the populations on the three media were the same was beyond the scope of this study. The problem of determining the effect

TABLE III

EFFECT OF HEAT IN PREPARATION OF SOIL-EXTRACT AGAR ON NUMBERS OF BACTERIA. THREE MEAN COUNTS, EACH FROM 10 REPLICATE PLATES FROM ONE AND THE SAME DILUTION OF SOIL

Soil*	Heated	Filtered	½ H + ½ F
1	90	55	90
6	137	70	119
8	66	45	64
9	54	38	52
10	100	54	91

*1, 6, 8: See footnotes Table II.
9: Clay, virgin forest, Red River Association.
10: Garden.

*The amount per liter previously found necessary with extracts from this soil to give pH 7.0 in the complete medium.

of heat in releasing specific growth factors from soil, or in inactivating others, would involve detailed nutritional studies on large numbers of isolates from each medium. Lochhead and Chase (4) reported that only one of 63 cultures, known to require soil extract, produced maximum growth in a basal salts, glucose, yeast extract medium in which soil-extract ash replaced soil extract. This may be accepted as indicating that extreme heat inactivates most of the growth factors present in soil extract.

Dried Soil Extract

Soil extract, in liter quantities with dipotassium acid phosphate added, was dried at 60° C. for 4 days and stored at laboratory temperature and humidity for periods ranging from 13 to 25 weeks. Agar was not added before drying because of the problem of drying and pulverizing, and it was considered that for this study the addition of agar at the time of testing was simpler. For each test a dried extract was suspended in distilled water by boiling for a brief period. After filtering to remove sediment, agar was added and the medium sterilized and used along with freshly prepared soil-extract agar made from an aliquot of the soil from which the dried extract had been prepared. Comparative counts on 13 samples representing eight soils are presented in Table IV. The freshly prepared extract gave higher counts in 11 of the 13 trials.

TABLE IV

EFFECT OF DRYING SOIL EXTRACT ON NUMBERS OF BACTERIA DEVELOPING ON SOIL-EXTRACT AGAR. TWO MEAN COUNTS, EACH FROM 10 REPLICATE PLATES FROM ONE AND THE SAME DILUTION OF SOIL

Soil*	Weeks dried	Dried extract	Fresh extract
1*a*	13	58	78
b	19	35	47
c	21	61	125
d	25	54	83
2*a*	13	100	124
b	19	47	73
3*a*	13	42	37
b	19	21	21
4	25	63	143
5	25	86	172
6	21	70	90
7	21	50	56
8	21	39	64

*See footnotes Table II.

It should be noted that this finding would not necessarily apply to soil-extract agar prepared by methods used for other dehydrated media. The extract was dry, whereas previously opened commercial dehydrated nutrient broth contained 5.0% moisture, nutrient agar 5.0%, yeast extract 9.8%, and malt extract 19.0%.

Dipotassium Acid Phosphate in Soil-Extract Agar

Soil-extract agar prepared as outlined above with 0.02% dipotassium acid phosphate was used along with soil-extract agar without it to obtain counts of bacteria in five soils. Higher concentrations were not investigated because even though accurate information on the requirements of potassium and phosphorus is not available the hundred or fewer bacteria in 1 ml. of the dilution plated could not assimilate the amount of these elements in 12 ml. medium in the plate. The results are presented in Table V. The medium with dipotassium acid phosphate gave higher counts in all trials.

TABLE V

EFFECT OF 0.02% K_2HPO_4 IN SOIL-EXTRACT AGAR ON NUMBERS OF BACTERIA DEVELOPING ON PLATES. TWO MEAN COUNTS, EACH FROM 10 REPLICATE PLATES FROM ONE AND THE SAME DILUTION OF SOIL

Soil*	With K_2HPO_4	Without K_2HPO_4
1	88	61
6	111	84
8	58	42
9	50	34
10	35	20

*1, 6, 8: See footnotes Table II.
9, 10: See footnotes Table III.

The Preparation of Soil-Extract Agar

1. Select a field soil of high fertility.

2. Use 500 g. soil and the amount of water, predetermined for the soil used, necessary to yield 1000 ml. extract. That amount of water will depend upon the moisture-holding capacity and the moisture content of the soil. It will vary from 1200 to 1500 ml. This eliminates the need for adding water after filtration, which would lower the concentration of extractives. It yields the maximum proportion of extractives obtainable by one extraction from the amount of soil used. In 16 trials with three soils, when 1000 g. soil and 1000 ml. water were used, the volumes of extract recovered ranged from 270 to 480 ml., and contained only 27 to 48% of the extractives. In a single trial, when 500 g. soil and 1200 ml. water were used, the volume of extract was 580 ml., and 58% of the extractives were recovered. In six trials with different soils, when 500 g. soil and 1300 ml. water were used, the extractives represented from 53 to 62%. When in a subsequent trial 500 g. soil and 1500 ml. water were used 1000 ml. extract and 67% of the extractives were recovered. Undoubtedly, this reasoning established the proportion of soil and water used by Smith and Worden (7).

3. Heat in an autoclave at 121° C. for 30 minutes.

4. Filter through filter paper or cloth. Refilter the first cloudy portion of the filtrate by returning it to the same filter. If a precipitant has been found necessary for the soil used add 0.5 g. calcium sulphate or 0.5 g. calcium carbonate after heating and let stand for 5 minutes before filtering.

5. To 1000 ml. filtrate add 0.2 g. dipotassium acid phosphate.
6. Add 15 g. agar.
7. Heat to dissolve.
8. Adjust to pH 6.8.
9. Filter through cotton, and dispense in 100 ml. quantities.
10. Sterilize at 121° C. for 20 minutes.
11. Temper to 50° C. and use, or store, tightly sealed, at 5° to 10° C. for later use.

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THE EFFECTS OF PROTAMINE AND HISTONE ON ENTEROBACTERIAL LIPOPOLYSACCHARIDES AND HEMOLYSIS[1]

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Abstract

The effects of protamine and histone, basic simple proteins, on enterobacterial lipopolysaccharides were studied, utilizing the enterobacterial hemolysis test as indicator system. The latter is based on the fact that these antigens become readily attached to sheep erythrocytes and thus endow the latter with a new serologic specificity. As a result, hemolysis occurs upon the addition of homologous bacterial antibodies and guinea pig complement. It was found that protamine inhibits enterobacterial hemolysis by interaction with the antigen and prevention of attachment of the antigen to red blood cells. Protamine in like concentrations does not inhibit hemolysis of lipopolysaccharide-modified red blood cells, nor does it affect red blood cells directly. The inhibitory effect of protamine is abolished by trypsin treatment of the lipopolysaccharide–protamine mixtures. Histone, too, inhibits enterobacterial hemolysis. Its mode of action, however, is different from that of protamine, in so far as treatment of red blood cells with histone prior to lipopolysaccharide modification causes inhibition of hemolysis, conceivably because this protein either blocks red blood cell receptors or otherwise interferes with subsequent hemolysis. Furthermore, histone–lipopolysaccharide complex becomes attached to erythrocytes; specific hemolysis ensues after treatment of the latter with trypsin. The results of this investigation are discussed with particular reference to their bearing upon the biologic activities of lipopolysaccharides, namely, cell affinity, toxicity, pyrogenicity, and alteration of host resistance, notably via the properdin system.

Introduction

Bacterial and tissue lipopolysaccharides are of current interest, since these substances nonspecifically alter host resistance to various infections and interact with properdin (27, 28, 29). Lipopolysaccharides from Gram-negative bacteria, which are integral parts of the somatic antigens (endotoxins), also are toxic and highly pyrogenic. In addition, they readily become attached to red blood cells of various animal species and thus endow the modified erythrocytes with a newly acquired serologic specificity. As a result, the erythrocytes are specifically agglutinated by homologous bacterial antiserum and red blood cells from certain animal species, notably sheep, are lyzed in the presence of antibody and guinea pig complement. In experiments with crude enterobacterial antigens and highly purified lipopolysaccharides, it was shown that hemagglutination and/or hemolysis of sheep erythrocytes are inhibited by lecithin, cholesterol, normal plasma (particularly fractions IV-1, IV-7, and albumin), certain polypeptide antibiotics, and by lipids obtained from serum (5, 20, 21, 24, 25). Similarly, agglutination of erythrocytes due to tuberculopolysaccharide is prevented by cephalin, lecithin, and extracts of red blood cell stromata, and mallein-conditioned agglutination by an extract

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obtained from ox erythrocytes (2, 3). Vogel (36) reported that bovine serum albumin (fraction V) inactivated the erythrocyte-sensitizing substances of both *Candida* and *Saccharomyces*. In view of the inhibitory effects of certain proteins and polypeptide antibiotics, the present study was undertaken to determine whether basic simple proteins, namely, protamine and histone, are similarly effective as inhibitors of bacterial hemagglutination and hemolysis and, if so, to analyze their mode of action. The results of this investigation are embodied in this report.

Material and Methods

Highly purified lipopolysaccharides from *Shigella sonnei* and *Escherichia coli* O111:B4, prepared by the phenol/water extraction method (37) were used. In addition, lipopolysaccharides of lesser purity from *E. coli* O127:B8 and O111:B4 were made available by Difco Laboratories through the courtesy of Dr. C. W. Christensen. Crude antigens were prepared as described previously (23). All materials were heated in boiling water for 1 hour to enhance erythrocyte modifying capacity (22). Specific antisera were obtained by immunization of rabbits. Lyophilized guinea pig serum (Carworth Farms, Inc., New City, N.Y.) was used as complement.

Two protamine preparations were employed, 1% protamine sulphate solution (Eli Lilly Company, Indianapolis, Indiana) and protamine sulphate (Nutritional Biochemicals Corporation, Cleveland, Ohio) of salmine origin. Histone (Nutritional Biochemicals Corporation, Cleveland, Ohio) was obtained from calf thymus nuclei. Lyophilized trypsin (Difco Laboratories, Detroit, Michigan) was dissolved in appropriate amounts of buffer solution to give a concentration of 10,000 μg./ml. This solution was stored in the refrigerator for a maximum of 6 days.

As diluent phosphate hemagglutination buffer (Difco Laboratories, Detroit, Michigan) (pH 7.3) was used throughout these experiments.

Enterobacterial hemagglutination and hemolysis and hemolysis inhibition tests were carried out according to the procedures developed in this laboratory (19, 22, 23), except for the inclusion of protamine, histone, and trypsin, as described in the text.

Results

In the first experiment the effect of protamine on *S. sonnei* hemolysis was determined. Mixtures of lipopolysaccharide (7.5 μg./ml.) and protamine sulphate in amounts of 2.5 to 0.15 μg./ml. were prepared. Buffer was used for control purposes. These mixtures were incubated in a water bath at 37° C. for 30 minutes and then employed for the treatment of sheep red blood cells. The suspensions were kept at 37° C. for 30 minutes, washed three times, and then added to *S. sonnei* antiserum in various dilutions and guinea pig complement. The resulting hemolysis was read after incubation for 30 minutes in a water bath at 37° C. The results are summarized in Table I.

TABLE I

EFFECTS OF PROTAMINE SULPHATE ON *S. sonnei* HEMOLYSIS

Hemolysis of red blood cells of sheep after treatment with mixture of *S. sonnei* lipopolysaccharide (7.5 μg./ml.) and various amounts of protamine sulphate

S. sonnei antiserum	Buffer	Protamine sulphate, μg./ml.				
		2.5	1.25	0.6	0.3	0.15
1:50	4	–	–	3	3	3
1:100	4	–	–	2	3	3
1:200	3	–	–	1	2	2
1:400	2	–	–	±	2	2
1:800	±	–	–	–	±	±
1:1600	–	–	–	–	–	–
0	–	–	–	–	–	–

NOTE: – = no hemolysis.
1 to 4 = various degrees of hemolysis.

Perusal of Table I shows that protamine in amounts of 1.25 μg./ml. prevented hemolysis completely, and smaller amounts (0.6 μg./ml.) proved to be partially inhibitory. Similar results were obtained with various other purified lipopolysaccharides and crude enterobacterial antigens. It is interesting to note, however, that larger amounts of protamine were required for inhibition of hemolysis when crude rather than highly purified lipopolysaccharides were employed. It should be mentioned also that protamine inhibited enterobacterial hemagglutination as well. No significant differences between the two protamine preparations were noted in this and the following experiments. It may be concluded, therefore, that protamine sulphate, a simple protein, inhibits enterobacterial hemagglutination and hemolysis.

To explain the mode of action of protamine, several possibilities were considered. (1) Protamine interferes with the attachment of lipopolysaccharide to the surface of red blood cells. (2) It destroys or alters the antigenic characteristics of the lipopolysaccharide, namely, the groupings reacting with the homologous bacterial antibodies. (3) Protamine blocks the receptors for the lipopolysaccharide on the surface of red blood cells. (4) Protamine inhibits the hemagglutination and hemolysis reactions per se.

If, in fact, protamine prevents the attachment of lipopolysaccharide to the surface of red blood cells, then, it should be ineffective as inhibitor of hemolysis of previously modified red blood cells. With these considerations in mind, the following experiment was set up.

Various amounts of *S. sonnei* lipopolysaccharide were used for modification of sheep red blood cells. The latter were washed three times and then treated with protamine in amounts of 2.5 μg./ml. and buffer, respectively. This amount of protamine proved to be effective as inhibitor of hemolysis when used together with the antigen for the treatment of erythrocytes (see Table I). The results of this experiment are presented in Table II.

Table II shows that protamine did not inhibit hemolysis of previously modified red blood cells. Particularly noteworthy is the fact that even with

TABLE II

EFFECTS OF PROTAMINE SULPHATE ON *S. sonnei* HEMOLYSIS

Hemolysis of red blood cells of sheep after consecutive treatments with *S. sonnei* lipopolysaccharide and protamine sulphate (2.5 μg./ml.)

S. sonnei antiserum	*S. sonnei* lipopolysaccharide, μg./ml.							
	5		2.5		1.25		0.6	
	Buffer	Protamine	Buffer	Protamine	Buffer	Protamine	Buffer	Protamine
1:50	3	3	2	2	1	1	±	±
1:100	2	2	2	2	±	±	–	±
1:200	2	2	1	1	–	–	–	–
1:400	1	1	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–

NOTE: –=no hemolysis.
1 to 4 =various degrees of hemolysis.

minimal amounts of *S. sonnei* lipopolysaccharide (1.25 μg./ml.) inhibition did not take place. This experiment indicates, furthermore, that protamine in the amounts used did not substantially alter the reactivity of the *S. sonnei* lipopolysaccharide with antibody, nor did it inactivate either bacterial antibody or complement. The conclusion that protamine in the concentrations and in the experimental procedure used does not substantially interfere with either antibody or complement is supported also by the observation that, as shown in parallel experiments, this protein does not inhibit hemolysis in the presence of sheep cell antibody (amboceptor) and guinea pig complement.

To ascertain whether protamine affects the erythrocyte receptors, the following experiment was set up. Sheep erythrocytes were treated with protamine in amounts of 6 to 50 μg./ml., followed by repeated washing and treatment with *E. coli* O127 lipopolysaccharide (50 μg./ml.). For control purposes, red blood cells were treated in the reverse order and also with mixtures of equivalent amounts of protamine and lipopolysaccharide. The hemolysis tests were carried out as described above. The results are recorded in Table III.

It can be seen that protamine treatment of the red blood cells did not interfere to a significant degree with subsequent hemolysis. In agreement with the previous experiment hemolysis was not inhibited by protamine treatment of previously modified sheep red blood cells. In contrast, mixtures of protamine (12 μg./ml. and up) and lipopolysaccharide completely prevented hemolysis. It may be concluded, therefore, that the major effect of protamine in the enterobacterial hemolysis system is directed toward the lipopolysaccharide and not toward the red blood cells.

The experiments presented thus far indicate that protamine interacts with enterobacterial lipopolysaccharides. To ascertain whether this interaction is reversible and leaves the lipopolysaccharide unaltered with respect to affinity for red blood cells and reactivity with homologous antibody, the following experiment was carried out. *E. coli* O127 lipopolysaccharide (25 μg./ml.) was mixed with protamine in amounts ranging from 0.3 to 5 μg./ml. and buffer, respectively. The mixtures were incubated in a water bath at 37 °C.

TABLE III

THE EFFECTS OF PROTAMINE ON *E. coli* HEMOLYSIS

Lysis of red blood cells of sheep after treatment as follows:

A. With lipopolysaccharide (50 μg./ml.) and various amounts of protamine.
B. With lipopolysaccharide (50 μg./ml.) followed by various amounts of protamine.
C. With various amounts of protamine followed by lipopolysaccharide (50 μg./ml.).

E. coli O127 antiserum	μg./ml. protamine														
	Treatment A					Treatment B					Treatment C				
	50	25	12	6	0	50	25	12	6	0	50	25	12	6	0
1:100	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4
1:200	–	–	–	3	4	4	4	4	4	4	3	3	3	4	4
1:400	–	–	–	1	3	3	4	4	4	3	3	3	3	3	2
1:800	–	–	–	–	2	2	2	2	2	2	2	2	2	2	2
1:1600	–	–	–	–	–	1	1	–	–	–	–	–	–	–	–
1:3200	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

NOTE: – =no hemolysis.
1 to 4 =various degrees of hemolysis.

for 30 minutes. One aliquot was then mixed with trypsin (50 μg./ml.), the other with an identical amount of buffer. The mixtures were incubated at 37° C. for 30 minutes and then used for modification of sheep erythrocytes in the usual manner. The suspensions were washed thrice. The treated suspensions were tested with homologous bacterial antibodies and guinea pig complement. The resulting hemolysis is recorded in Table IV.

TABLE IV

THE EFFECTS OF PROTAMINE ON *E. coli* HEMOLYSIS

Lysis of red blood cells of sheep after treatment as follows:

A. With a mixture of lipopolysaccharide (25 μg./ml.) and various amounts of protamine.
B. With a mixture of lipopolysaccharide (25 μg./ml.), various amounts of protamine, and trypsin (50 μg./ml.).

E. coli O127 antiserum	μg./ml. protamine											
	Treatment A						Treatment B					
	5	2.5	1.25	0.6	0.3	0	5	2.5	1.25	0.6	0.3	0
1:100	–	–	4	4	4	4	4	4	4	4	4	4
1:200	–	–	2	3	4	4	4	4	4	4	4	4
1:400	–	–	1	2	3	3	3	3	3	3	3	3
1:800	–	–	–	–	1	1	2	1	2	2	1	2
1:1600	–	–	–	–	–	–	–	–	–	–	–	–
1:3200	–	–	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–	–	–

NOTE: – =no hemolysis.
1 to 4 =various degrees of hemolysis.

Table IV shows that, as expected, protamine inhibited hemolysis. In contrast, trypsin treatment of the mixture of lipopolysaccharide and protamine abolished this inhibitory effect. It is evident, therefore, that protamine

does not cause irreversible alteration of the lipopolysaccharide with respect to both affinity for erythrocytes and reactivity with antibody. It should be emphasized that trypsin in the concentrations used and under the conditions of these experiments did not cause a Thomsen–Friedenreich type of reaction.

Further proof of the thesis that protamine, by interaction with lipopolysaccharide, prevents attachment of the latter to erythrocytes was obtained in an experiment, based on the following reasoning. If the protamine–lipopolysaccharide complex became attached to erythrocytes, then, treatment of these red blood cells with trypsin should abolish the inhibitory effect of protamine and make hemolysis possible. To this end, sheep erythrocytes were treated with mixtures of *E. coli* O111 lipopolysaccharide (5 μg./ml.) and protamine (31 and 62 μg./ml.) or buffer for control purposes. The suspensions were incubated, washed, and then treated with trypsin (200 μg./ml.) and buffer, respectively. The hemolysis tests were carried out in the usual manner. The results are summarized in Table V.

TABLE V

EFECTS OF TRYPSIN ON PROTAMINE–LIPOPOLYSACCHARIDE-TREATED ERYTHROCYTES

E. coli hemolysis after treatment of erythrocytes as follows:

A. With various amounts of protamine and lipopolysaccharide (5 μg./ml.) followed by trypsin (200 μg./ml.).
B. With various amounts of protamine and lipopolysaccharide (5 μg./ml.) followed by buffer.

E. coli O111 antiserum	μg./ml. protamine					
	Treatment A			Treatment B		
	62	31	0	62	31	0
1:50	±	±	4	–	–	4
1:100	–	–	4	–	–	4
1:200	–	–	4	–	–	4
1:400	–	–	4	–	–	4
1:800	–	–	3	–	–	3
1:1600	–	–	2	–	–	1
1:3200	–	–	–	–	–	–
0	–	–	–	–	–	–

NOTE: – =no hemolysis.
1 to 4 =various degrees of hemolysis.

Table V shows that only minimal or questionable hemolysis occurred after trypsin treatment of lipopolysaccharide–protamine-exposed erythrocytes. It is reasonable to conclude on the basis of this and similar experiments that the major amount of lipopolysaccharide used for treatment of sheep red blood cells in the presence of protamine does not become attached to red blood cells. That trypsin in identical amounts renders lipopolysaccharide effective when used for the treatment of lipopolysaccharide–protamine mixtures prior to modification of erythrocytes has been documented above.

Hemolysis inhibition tests revealed that mixtures of lipopolysaccharides and protamine are effective as antigens neutralizing homologous antibodies, although slightly less so than lipopolysaccharides alone. This observation

indicates that protamine does not substantially interfere with the capacity of the antigen to react wih the corresponding antibody.

Studies on the effects of histone on enterobacterial hemolysis revealed results similar to, but not identical with, those obtained with protamine. Similarities were the following. (1) Histone, like protamine, upon addition to enterobacterial lipopolysaccharides inhibited subsequent hemolysis. In titration experiments it was found that 62 μg./ml. and more of histone caused complete and smaller amounts (31 μg./ml.) partial inhibition of hemolysis. With an increase in the amounts of lipopolysaccharide a correspondingly larger amount of histone is required. (2) A second similarity between histone and protamine was found in the observation that inhibitory amounts of histone failed to cause marked inhibition of hemolysis of previously lipopolysaccharide-modified sheep red blood cells. In a few instances a minimal inhibitory effect was noted. (3) Trypsin treatment of histone–lipopolysaccharide mixtures abolished the inhibitory effects of histone, in accordance with the results obtained with protamine.

A marked difference in the action of these two proteins was detected in studies on the effects of these proteins on red blood cells, as the following experiment reveals. Sheep red blood cells were treated with histone (8 to 125 μg./ml.) and with buffer as a control. One aliquot was then mixed with trypsin (200 μg./ml.), the other with a corresponding amount of buffer. The suspensions were washed and treated with *E. coli* O111 lipopolysaccharide (5 μg./ml.). In parallel, sheep erythrocytes were treated with mixtures of histone and lipopolysaccharide in corresponding amounts. The hemolysis tests were carried out in the usual fashion. The results are summarized in Table VI.

TABLE VI

THE EFFECTS OF HISTONE ON *E. coli* HEMOLYSIS

Lysis of red blood cells of sheep after treatment as follows:

A. With various amounts of histone, followed by trypsin (200 μg./ml.), followed by lipopolysaccharide.
B. With various amounts of histone, followed by buffer, followed by lipopolysaccharide.
C. With a mixture of various amounts of histone and lipopolysaccharide.

E. coli O111 antiserum	μg./ml. histone											
	Treatment A				Treatment B				Treatment C			
	125	31	8	0	125	31	8	0	125	31	8	0
1:50	2	4	4	4	–	2	4	4	–	2	4	4
1:100	2	4	4	4	–	2	4	4	–	2	4	4
1:200	2	4	4	4	–	1	4	4	–	–	4	4
1:400	2	4	4	4	–	–	4	4	–	–	2	4
1:800	2	3	3	4	–	–	2	3	–	–	1	3
1:1600	–	1	2	1	–	–	–	1	–	–	–	1
1:3200	–	–	1	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–	–	–

NOTE: – = no hemolysis.
1 to 4 = various degrees of hemolysis.

Table VI shows that histone in amounts of 125 μg./ml., when mixed with lipopolysaccharide prior to treatment of the erythrocytes, completely prevented hemolysis; 31 μg./ml. proved to be partially inhibitory (column 3). A similar result was obtained when erythrocytes were first treated with histone and subsequently with lipopolysaccharide (column 2). This inhibitory effect was substantially reduced by trypsin (column 1). These data suggest that histone becomes attached to the surface of erythrocytes and thus blocks the attachment of lipopolysaccharide and/or subsequent hemolysis. It should be noted that the larger amounts of histone caused some agglutination, which disappeared upon repeated washings.

The question arises as to whether or not histone carries lipopolysaccharide to the surface of erythrocytes. To elucidate this problem, in the following experiment advantage was taken of the proteolytic activity of trypsin.

Sheep red blood cells were treated with mixtures of *E. coli* O111 lipopolysaccharide (2.5 μg./ml.) and various amounts of histone (5 to 135 μg./ml.). These mixtures had been kept at 37° C. for 30 minutes. One aliquot of each red blood cell suspension was then treated at 37° C. for 30 minutes with trypsin (1000 μg./ml.), the other with buffer. The hemolysis test was carried out as described above. The results are recorded in Table VII.

TABLE VII

EFFECT OF TRYPSIN ON HISTONE–LIPOPOLYSACCHARIDE-TREATED ERYTHROCYTES

E. coli hemolysis after treatment of erythrocytes as follows:

A. With a mixture of lipopolysaccharide (2.5 μg./ml.) and various amounts of histone followed by buffer.
B. With a mixture of lipopolysaccharide (2.5 μg./ml.) and various amounts of histone followed by trypsin (1000 μg./ml.).

	μg./ml. histone									
	Treatment A					Treatment B				
E. coli O111 antiserum	135	45	15	5	0	135	45	15	5	0
1:50	–	–	3	4	4	4	4	4	4	4
1:100	–	–	2	4	4	4	4	4	4	4
1:200	–	–	1	3	4	4	4	3	4	4
1:400	–	–	–	2	3	3	4	–	4	3
1:800	–	–	–	–	1	2	2	–	1	1
1:1600	–	–	–	–	–	–	–	–	–	–
1:3200	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–

NOTE: – = no hemolysis.
1 to 4 = various degrees of hemolysis.

It can be seen from Table VII that the inhibitory effect of histone was negated by the proteolytic enzyme, indicating that lipopolysaccharide was present on the erythrocytes. Of particular significance is the observation that hemolysis was stronger when larger (45 μg./ml.), highly inhibitory rather than smaller (15 μg./ml.), minimally inhibitory amounts of histone were employed.

These findings contrast sharply with the data obtained with protamine (see Table V). It should be pointed out that these differences between histone and protamine were observed in repeated, parallel experiments. It may be concluded that the histone–lipopolysaccharide complex becomes attached to erythrocytes and that larger amounts of histone apparently carry more antigen to the red blood cells than smaller amounts. The mechanism of the inhibition of hemolysis by histone present on the erythrocytes remains to be determined.

Discussion

The present study has revealed that the simple basic proteins protamine and histone interfere with enterobacterial hemolysis. Study of the mode of action of these inhibitors was facilitated by the use of the proteolytic enzyme trypsin. The results strongly suggest that protamine interacts with enterobacterial lipopolysaccharide and that the resulting complex is not, or only to a minimal degree, adsorbed to the surface of red blood cells. Trypsin treatment of the complex restores the red cell modifying capacity of the lipopolysaccharides, indicating that protamine does not alter either red cell affinity or antibody reactivity of the antigen. Histone, on the other hand, was shown to become attached to red blood cells as well and thus, either directly or indirectly, to contribute to the inhibitory effect on enterobacterial hemolysis. Gelatin (up to 1000 μg./ml.), on the other hand, did not inhibit enterobacterial hemolysis.

Fischer and associates (7, 8) found that both protamine and histone enter ascites tumor cells under appropriate conditions and cause morphologic and biochemical changes. A comparative investigation on the protamine and histone concentrations in tumor and red blood cells in the presence and absence of enterobacterial lipopolysaccharides appears to be indicated. In this connection attention should be called to the effect of protamine on sensitized and nonsensitized red blood cells. Roth and Frumin (32) found that normal red blood cells are agglutinated by protamine in a concentration of 10,000 μg./ml. but not of 800 μg./ml., whereas antibody-sensitized erythrocytes were agglutinated by protamine in the lower concentration. The mechanism of this reaction remains to be elucidated. The concentration of protamine used in the present study did not cause visible agglutination.

The interaction of protamine and lipopolysaccharide, resulting in formation of a complex, is in agreement with the previously reported finding of lipopolysaccharide–protein complexes. Lüderitz and associates (15, 16) showed that lipopolysaccharide complexes with casein and that the complexed casein is no longer precipitated by acetic or even trichloracetic acid. Similar complexes are formed with other proteins and certain lipids, such as cholesterol. Certain polypeptide antibiotics, notably neomycin and polymyxin B, also inhibit enterobacterial hemolysis and conceivably complex with these antigens (20). Stauch and Johnson (35) reported that incubation at 37° C. of *Salmonella typhosa* in serum devoid of antibodies resulted in a substantial loss of endotoxin, as measured by precipitating activities. It will be interesting to learn whether

the endotoxin can be freed from the serum factor by proteolytic enzymes and whether a relationship exists between precipitating and erythrocyte-modifying capacity of the serum-treated lipopolysaccharide. It was previously reported that certain, but not all, plasma fractions inhibit enterobacterial hemagglutination (24). In this connection it is of interest to refer to the observation of Rheins and Thurston (31), who demonstrated that normal serum of rabbit or human origin elutes BCG extract antigen from sheep erythrocytes. Albumin is responsible for this effect. Old tuberculin, on the other hand, is not eluted under similar conditions. It remains to be seen whether protamine and histone are capable of causing dissociation of lipopolysaccharide from erythrocytes under suitable conditions.

The effects of all the afore-mentioned inhibitors of enterobacterial hemagglutination and hemolysis should be studied by means of radioactively labeled antigen. Lüderitz and associates (17) have shown that this procedure is feasible, and they have confirmed the thesis, based on serologic evidence, that certain untreated enterobacterial lipopolysaccharides are far more poorly adsorbed by red blood cells than heated or NaOH-treated lipopolysaccharides.

The interaction of histone and red blood cells deserves further investigation. In particular, it is desirable to learn whether this simple protein blocks receptors of red blood cells for lipopolysaccharide and/or interferes with the hemagglutination and hemolysis reactions per se. In the search for an explanation of the findings one must keep in mind that histone does not markedly inhibit hemolysis of previously modified erythrocytes. Perhaps the lipopolysaccharide–histone complex becomes located on the erythrocyte in an area not available for the hemolysis reaction and trypsin treatment makes possible a change in location of the antigen. Alternatively, the histone present in the immediate vicinity of the antigen interferes with subsequent hemagglutination or hemolysis. In this connection, attention may be called to the observation that, in contrast to modified red blood cells from various warm-blooded animal species, those from alligator and axolotl are not agglutinated by enterobacterial antibodies. Studies of the reasons for these negative results revealed that a protein or protein-like substance on the surface of the latter erythrocytes interferes with the agglutination reaction per se, and not with the attachment of the lipopolysaccharide (18). The relationship, if any, between these phenomena is not yet clear.

Investigations on the interaction between enterobacterial lipopolysaccharides and various chemical substances are of interest also because of the biologic activities of these antigens. It is now clearly established that these lipopolysaccharides are highly toxic, extraordinarily pyrogenic, and profoundly affect the nonspecific resistance of certain animals to a variety of infections. The effects of protamine and histone on these reactions remain to be determined.

The question presents itself as to whether protamine and histone alter the interaction between lipopolysaccharides and properdin. Such an investigation was planned in collaboration with Dr. Louis Pillemer, but, owing to his untimely death, could not be completed. Dr. Jack Pensky of the Western

Reserve University, Cleveland, Ohio, was kind enough to carry out a pilot experiment (26). The result indicates that protamine appears to have a definite effect on the ability of *Pasteurella pseudotuberculosis* lipopolysaccharide to inactivate or complex with properdin. The possibility may be explored whether lipopolysaccharide–protamine complex can be used for increasing resistance through elevation of properdin level or other as yet poorly understood mechanisms, without causing an initial decrease of resistance. It is conceivable that such a complex also may have a more prolonged effect than lipopolysaccharide alone.

The interaction of protamine and histone with lipopolysaccharides also may have bearing on the pyrogenicity and toxicity of the latter. The pyrogenicity of crude culture filtrate and purified lipopolysaccharide is neutralized by fresh human serum, as reported by Hegemann (9, 10, 11). Rall *et al.* (30) found that fresh rabbit serum is likewise effective. Cluff and Bennett (4) showed that the ability of normal serum to inhibit endotoxin fever is, in part at least, dependent upon the properdin system. Ho and Kass (12) showed that crude endotoxin, in contrast to highly purified endotoxin obtained by Boivin's trichloracetic acid extraction method, was attenuated by human plasma fractions III and IV (Cohn). Kelly and co-workers (13) observed that rabbit serum reduced the lethality of crude, but not of purified, bacterial polysaccharide. Landy and associates (14) recently reported that fresh human serum inhibits the capacity of endotoxic polysaccharide to produce hemorrhagic necrosis in tumor and the Shwartzman reaction. The active factor in serum was not identified. This effect on tumor by human serum does not parallel the findings of Kelly *et al.* (13), who observed no inhibition of tumor-necrotizing activity of bacterial polysaccharide by fresh rabbit serum. Identification of the serum components responsible for these effects remains to be accomplished, and the antitoxic and antipyretic activity of protamine and histone should be investigated.

Irreversible, experimental shock is due to exogenous bacterial endotoxin, and nonabsorbable antibiotics, such as polymyxin B and neomycin, effectively prevent this syndrome, according to the investigations of Schweinburg, Fine, and their associates (33, 34). The possibility may be considered that complex formation between the lipopolysaccharide endotoxin and the antibiotics is responsible, to some extent, for this effect. The question arises also whether protamine or histone are likewise active.

Of particular interest is the fact that the basic protamine (6) and the basic antibiotics (polymyxin B and neomycin) all possess antibacterial potency and block the reactive groupings of bacterial lipopolysaccharides, which otherwise become attached to erythrocyte receptors. A systematic search for, or synthesis of, other basic proteins or polypeptides may reveal even more potent antibacterial agents and/or inhibitors of lipopolysaccharide endotoxins, substances which may be also less toxic for higher animals. It should be emphasized that different parts of the lipopolysaccharides are responsible for various biologic activities, namely, antigenicity, cell affinity, pyrogenicity,

and toxicity. This conclusion is based on observations to the effect that various physical and chemical treatments of these lipopolysaccharides affect the biologic activities differently (22). Therefore, it is entirely possible that various inhibitors of one biologic activity of the lipopolysaccharides are not necessarily equally effective against other activities.

Bazin and Delaunay (1) reported that, among other substances, typhoid lipopolysaccharide combines with collagen A. The question presents itself as to whether protamine, histone, and other inhibitors of enterobacterial hemolysis also interfere with this reaction and what relationship, if any, such interactions have with regard to the pathogenesis of collagen diseases. These and other problems await elucidation by further investigations.

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LOCALIZATION OF A TOXICITY FOR SILKWORM LARVAE IN THE PARASPORAL INCLUSION OF BACILLUS CEREUS VAR. ALESTI[1]

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Abstract

The parasporal protein inclusion of *Bacillus cereus* var. *alesti* accounts for one third of the dry weight of the washed products of sporulation. Although the spore:crystal ratio is 1:1, the weight ratio is 1.5:1. Like other parasporal crystals, those of var. *alesti* are swollen but not dissolved by alkalinity up to a critical pH (11.5–11.8); above this pH, dissolution occurs. Toxicity tests on extracts made at increasing pH levels indicated that the crystal protein itself is the toxic agent and not the carrier of it. The protein of actively dividing cells and of spores was non-toxic. Crystal-forming cells contained a toxic protein which would dissolve at a lower pH than would the free crystal.

Introduction

During sporulation each cell of *Bacillus cereus* var. *alesti*, like other insect pathogenic variants of *B. cereus* (var. *sotto* and var. *thuringiensis*) produces a bipyramidal crystal which approaches the spore in size (15). As *B. cereus* var. *thuringiensis* was the first of these variants to be isolated and described (7) it has enjoyed the distinction of being named as a separate species (6). However, the general characteristics of these three insect pathogens (16, 17, 18, 2) as well as a chemical and morphological comparison of their spores* (10) indicate that they are, and should be named as was suggested by Smith, Gordon, and Clark for *B. thuringiensis* (14), as variants of *B. cereus* Franklin and Franklin. In fact, mutants of these organisms which are similar to *B. cereus* strains in that they do not form crystals can be readily isolated from cultures of these crystal-forming variants. Moreover, Toumanoff (20) has recently reported that an ordinary *B. cereus* strain may become crystallophoric and virulent after several transfers by injection through the body cavity of *Galleria melonella* larvae.†

The redescription of one of these organisms by Hannay in 1953 and particularly his correlation of crystal formation with toxicity (11) awakened interest in these variants. Angus (1) soon demonstrated the toxic effects of the crystalline inclusions of *B. cereus* var. *sotto* (*B. sotto* Ishiwata) for silkworms and a preliminary report of the present results with *B. cereus* var. *alesti* crystals also appeared (19).

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Contribution from the Department of Bacteriology and Immunology and the Department of Biochemistry, University of Western Ontario, London, Canada, and the Institut Pasteur, Service de Pathologie des Insectes, Paris, France.
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*Fitz-James, P. C. and Young, I. E. To be published.
†Le Coroller (to be published) working in the Pasteur Institute has recently obtained similar results using other strains of saprophytic *B. cereus* than that used by Toumanoff.

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The results of Angus (3) indicate that the toxic material may be extracted from the crystal by alkali while the chemical data suggest the crystal is an aggregate of a single protein species (4, 12). Isolated crystals of *B. cereus* var. *sotto* and *alesti* like those of var. *thuringiensis* have been found to swell yet show no loss of nitrogen on treatment up to a critical pH (11.5–11.8). Beyond this, however, dissolution takes place (12). Excess alkali or local high-concentration effects of alkali could cause damage to the crystal protein, appear to extract an active toxin, and leave a non-toxic damaged residue such as Angus found. In the work reported here, the crystals were sequentially extracted at higher pH levels up to a pH of 12.2 and the toxicity of these extracts or their precipitated proteins compared. In addition the toxicity of alkali-extracted proteins of vegetative cells and cells in the later stages of spore and crystal formation are compared. The results indicate that the crystal protein is the toxin.

Materials and Methods

The organism used throughout was *B. cereus* var. *alesti*. The cultures were grown on the agar medium of Howie and Cruickshank as described previously (9).

Centrifugation was carried out in a Servall (SS-1) centrifuge. The pH measurements were done with a glass electrode pH meter (Beckman model G) standardized against a buffer (pH 4.0, 7.0, or 10.0) nearest the range being measured.

Total phosphorus was estimated by the method of King (13) as modified previously (9). Total nitrogen was determined in duplicate by micro-Kjeldahl procedure employing a micromodification of the ashing procedure of Beet (5). Ammonia in the digested sample was distilled from a Markham apparatus into boric acid (8).

The methods for preparing spores and crystals and the fractions thereof for insect testing are described below.

*Methods of Testing Preparations for Toxicity**

Larvae of *Bombyx mori* in the fourth and fifth instar were used. Material to be tested was applied to the mulberry leaves by two methods: (1) the substance was dissolved in 0.1 *N* NaOH at a concentration of 1 mg./ml. and leaves were dipped in this solution or dilution of it; or (2) the substance was suspended in water again at 1 mg./ml. and this or dilutions spread on the leaf surface. All preparations except A_3-7, which left a small amount of residue, readily dissolved in 0.1 *N* NaOH. All were well accepted by the fasted larvae. From 12 to 50 larvae were used for each preparation and received only one toxic meal. Subsequent meals were composed of normal mulberry leaves.

A comparison of the two methods of applying toxic materials to leaves was made using separated crystals (A_3-1). Crystals dissolved in 0.1 *N* NaOH and

*The experiments on silkworms were made in 1955 during the stay of one of us (C.T.) in Sericicultural Station of Ales (Gard). Thanks are expressed to Mr. Shenk, Director of this station, and Dr. Vago, Chief of the Pathology Division for providing silkworms.

suspended at 1 mg./ml. were tested after water dilutions of 1/100, 1/200, 1/400, and 1/1000. Although not quantitative this trial indicated that dilutions of water-suspended crystals were some two to three times more toxic than those dissolved first in alkali and then diluted. This evidence of alkali damage to crystal toxin prompted the use of suspensions for comparisons of toxicity.

Experimental and Results

The Yield of Crystals by a Culture of B. cereus var. alesti

Table I records the results of a study of the total yield of vegetative debris, spores, and crystals recovered from a completely sporulated culture (4 days on the agar of Howie and Cruickshank). Vegetative debris were separated out and repeatedly washed in water by differential centrifugation until free of spores and crystals. The crystals in the washed spore–crystal mixture were separated from the spores by dissolution in 0.1 *N* NaOH. The protein precipitated from this alkali extract at pH 4.5 was washed with water then weighed to give a measure of the crystal yield. The washed spores were also weighed. Although in the sporulating cells the spore:crystal ratio is approximately 1:1 (Fig. 1), the weight ratio of these two products in the sporulated culture of *B. cereus* var. *alesti* is approximately 1.5:1.

Preparations of Spores and Crystals

These were made from 4-day-old cultures. For good yields the growth of 10–12 Povitsky bottles was harvested and cleaned as described previously. Refractile spore–crystal mixtures were obtained by repeated centrifugation and resuspension in saline. After each centrifugation a fluffy top of vegetative debris was removed from a bottom creamy layer. For maximum yield, the top layers were repeatedly resuspended and recentrifuged till practically free of spores and crystals. Progress of the washing procedure was followed in air-mounted dried nigrosine smears. After 7 to 12 such saline or water washes a pellet composed entirely of spores and crystals was obtained. Such mixtures were used for preparing pure crystals by two methods, one based on spore disruption, the other, solely on differential centrifugation. The mechanical disruption of spores already described (10) for the preparation of crystals of *B. cereus* var. *thuringiensis* was used here to prepare the crystals for preparations A_1 and A_3-3 to A_3-7.

TABLE I

DISTRIBUTION OF CELL DEBRIS, SPORES, AND CRYSTALS IN A COMPLETELY SPORULATED CULTURE OF *B. cereus* VAR. *alesti*

	Yield	
	g./500 cm.² agar surface	% total yield
Vegetative cell debris	0.052	16
Spores	0.173	52
Alkali soluble protein (crystals)	0.109	32

Unlike the *thuringiensis* and *sotto* variants, a spore–crystal mixture of var. *alesti* would show partial separation of crystals from whole spores by differential centrifugation of saline suspensions. When free of cell debris, the spore–crystal pellet showed two distinct layers and a comparison indicated a slightly increased proportion of spores in the top layer. After some 30 cycles or resuspension, centrifugation, and separation, crystals of var. *alesti* were obtained almost free of spores (A_2). These formed a non-flocculent suspension in water (Fig. 2). The yield of crystals (mg./500 cm.^{2} agar surface) was 58 for the method of spore disruption and 50 for that of mechanical separation.

A comparison of the nitrogen and phosphorus content of the crystals separated from whole spores and those separated from disrupted spores showed little difference between these products (Table II). The higher phosphorus content of preparation A_2 indicates a slightly greater degree of spore contamination. Their toxicity to larvae was likewise similar (Table IV).

TABLE II

A COMPARISON OF THE NITROGEN AND PHOSPHORUS CONTENT OF CRYSTALS SEPARATED BY DIFFERENTIAL CENTRIFUGATION FROM DISRUPTED AND FROM WHOLE SPORES

	Method of separation	% P	% N
A_1 crystals	Spore disruption followed by differential centrifugation	0.010	14.3
A_2 crystals	Differential centrifugation from whole spores	0.028	14.4

Titration of Alesti Crystals with Dilute Sodium Hydroxide

In a study of the effect of dilute alkali on the solubility of purified crystals of *B. cereus* var. *thuringiensis*, Hannay and Fitz-James (12) found that in spite of marked swelling in alkali, solution of the protein crystal did not occur until the pH was elevated above 11.8. Even then, some 84% of the crystal protein at pH 12.2 could be brought down at 10,000 *g*. A similar titration was carried out at 29° C. on a small batch (25 mg.) of a pure preparation of *alesti* crystals (A-1). The alterations in morphology were followed in nigrosine smears. First signs of swelling occurred when the pH reached 10.52. Such crystals now formed a soft, yellow-tinged pellet but were still readily resuspended in water. They were re-extracted at pH 10.3, and the pooled washes respun at 10,000 *g* before nitrogen analysis. Only a trace of the crystal nitrogen was recovered in this wash (Table III).

The pH of the resuspended crystal residue was next slowly raised with 0.02 *N* alkali to 11.0. Swelling became more marked and the soft, uniform, light-yellow pellet of swollen crystals which was recovered after centrifugation at 10,000 *g* could still be easily resuspended in water. The pH 11.0 supernatant likewise contained only a fraction of the total crystal nitrogen (Table III). When the resuspended pellet was treated with further alkali (0.1 *N* NaOH), the swollen crystals appeared to undergo some disintegration at pH 11.8

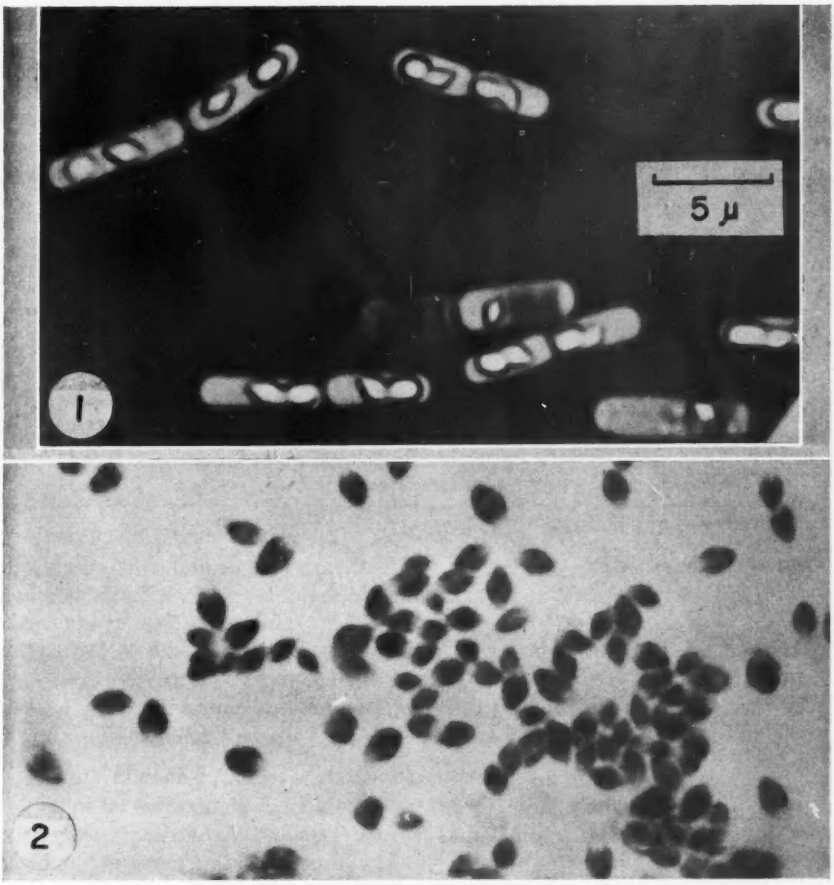

FIG. 1. Air-mounted nigrosine smear of a sporulated culture of *B. cereus* var. *alesti* prior to lysis showing the relative size of spore and crystal.

FIG. 2. Crystals of var. *alesti* separated from a spore–crystal mixture by differential centrifugation, stained with methyl green. Magnification as indicated.

to pH 12.0. The opaque, yellowish suspension began to clear and at pH 12.2 a slightly turbid solution remained. However, when this was centrifuged at 10,000 *g* for 10 minutes a thick gel-like pellet was recovered under a clear viscous solution. The gel pellet would no longer resuspend easily but when worked into water it formed a clear viscous suspension. From Table III it can be seen that only 9% of the crystal N was dissolved during this last pH rise. All of this dissolved nitrogen, however, could be recovered in the precipitate which formed when the pH was lowered to 4.9.

TABLE III

THE PERCENTAGE OF TOTAL CRYSTAL NITROGEN EXTRACTED FROM A PURE CRYSTAL SUSPENSION AT SUCCESSIVE pH INCREMENTS AND THE ASSOCIATED CRYSTAL MORPHOLOGY

pH increments of successive extractions	Morphology in nigrosine smears	% total nitrogen extracted
6.0–10.5	Loss of refractility and slight swelling	0.53
10.5–11.0	Marked swelling	0.65
11.0–11.8	Some disintegration of swollen crystals	–
11.0–12.2	Marked loss of crystal structure	9.0

Preparation of Crystal Fractions for Toxicity Tests

A similar titration to that described in the previous section on A-1 crystals was conducted on a larger batch of purified crystals (A_3) and these fractions subjected to toxicity tests.

The *pH 10.8 wash* of A_3 crystals caused a general swelling of the crystals. However the extract, after centrifugation, failed to yield a precipitate at pH 4.5. This fraction was shipped for testing as a liquid (A_3-3). It was practically devoid of toxicity (Table IV).

The pH 11.8–12.2 Extract of A_3 Crystals

The pellet of crystals remaining after the pH 10.8 wash was now treated with more alkali (0.1 *N* NaOH). At pH 11.5 definite buffering was encountered and crystals appeared more swollen. The pH was raised to 12.2. After centrifugation at 10,000 *g*, again a jelly-like pellet of crystals was obtained under a clean, slightly viscous supernatant. This supernatant was recentrifuged then precipitated at pH 5.0. The precipitate was redissolved in dilute alkali, reprecipitated at pH 5.0, and freeze-dried to form preparation A_3-5. The supernatant from the first precipitation was also saved and freeze-dried (A_3-4). The toxicity extracted by the alkali was entirely located in the precipitated protein (compare A_3-4 and A_3-5, Table IV).

Crystal Gel Remaining after Alkali Dispersion

The major portion of the original crystals, still undissolved after repeated washing at pH 11.9–12.0, was washed twice with water. A wet (A_3-6) and a freeze-dried sample (A_3-7) were sent for testing. A_3-6 was spread on leaves as received; A_3-7 was applied in 0.1 *N* NaOH. By both procedures, this crystal residue was still highly toxic (Table IV).

TABLE IV

THE EFFECT OF FEEDING VARIOUS FRACTIONS OF *B. cereus* VAR. *alesti* TO SILKWORM LARVAE

Fraction number	Source and method of preparation	Description of preparation	Conclusion of feeding tests
A_1	Crystals completely separated from disrupted spores	Water suspension	Highly toxic
A_2	Crystals completely separated from whole spores mechanically	Water suspension	Highly toxic
A_3-3	pH 10.5 wash of partially purified crystals	A liquid (no precipitate at pH 4.5)	Slightly toxic (15% of larvae paralyzed in 24 hours)
A_3-4	Crystals, previously washed at pH 10.5, extracted at pH 11.8–12.0 = alkali extract of crystals, adjusted to pH 5.0 and centrifuged	Supernatant (freeze-dried)	No detectable toxicity
A_3-5		Residue: reprecipitated and washed (freeze-dried)	Toxic
A_3-6	Crystal gel remaining after alkali dispersion, water-washed	Wet sample	Highly toxic
A_3-7		Dry sample	Highly toxic
A_3-8	Spores repeatedly washed in alkali and acid	Water suspension	No immediate paralysis (20% dead in 16 hours, 50% dead in 50 hours)
A_3-9	A_3-8 spores completely disrupted	Freeze-dried powder	No effect on larvae
A_4-1	Growing vegetative cell protein extracted at pH 11–12	Freeze-dried at pH 4.5	Non-toxic
A_4-2	Sporulating cell protein extracted at pH 10.5	Freeze-dried at pH 4.5	Toxic

Spores for Toxicity Tests

These were obtained by dissolving the crystals from a spore–crystal mixture by washing with alkali (0.2 *N* NaOH) at a pH of 12.5. On centrifugation at 10,000 *g* creamy spores were sedimented as a bottom pellet under the swollen crystals. They were rewashed in 0.2 *N* NaOH, then several times in water or saline. As was found for *B. cereus* var. *sotto* (1) the spores of this variant showed no immediate toxic effect on silkworm larvae. But contrary to the earlier findings with var. *sotto* at 12 hours, these spores of var. *alesti* did show some deaths by 16 hours and by 50 hours 80% of the larvae were dead. The disrupted spores were both non-toxic and non-septicaemic (Table IV).

The Protein of Young Vegetative Cells (A_4-1)

Proteins extracted at pH 11.5 from young vegetative cells were also tested for toxicity. Cells for this material were scraped from the agar surface of four Povitsky bottles 12 hours after inoculation with a spored culture (incubation at 25° C.) and suspended in 200 ml. of heart infusion broth. After 2 hours' further incubation at 37° C., the culture of actively dividing cells was collected by centrifugation, washed once in water, and resuspended in 25 ml. H_2O. The pH of the suspension was raised to 11.5 at 25° C. for 15 minutes. After centrifugation the clear supernatant was adjusted to pH 4.5 with 0.1 *N* HCl and cooled at 2° C. The resulting precipitate was collected, then redissolved at pH 12. This solution was clarified by centrifugation, reprecipitated at pH 4.5 (at 2° C.), washed once with cold water, and lyophilized. This protein of growing cells showed no toxic effect on silkworms (Table IV).

The pH 10.8 Extractable Protein of Sporulating Cells (A_4-2)

Although the toxicity of the ripe separated crystals of var. *alesti* was largely insoluble at pH 10.8, it could be readily extracted from sporulating cultures at this pH (Table IV). This fraction (A_4-2) was prepared from a 2-day-old growth on the agar of Howie and Cruickshank. Such a culture contained only a few free spores and crystals; most of the cells were in the later stages of sporulation and forming both these inclusions. Two Povitsky bottles were harvested at this stage, the crop washed once in water, and once with saline. The pH of the suspended growth was raised to 10.8 with small additions of 0.1 *N* NaOH at 25° C. After centrifugation at 10,000 *g* for 5 minutes, the residue was rewashed at pH 10.8. The combined alkali supernatants were clarified by a repeated centrifugation at 10,000 *g*, after which the pH was lowered to 4.5 with 0.1 *N* HCl and the resulting precipitate collected, washed with ice-cold distilled water, and freeze-dried.

Discussion

From these tests it becomes apparent that the toxicity of the cultures of *B. cereus* var. *alesti* for silkworm larvae, like that of *B. cereus* var. *sotto* (1, 3), resides in the crystal rather than in the spore. Those crystals separated from disrupted spores had a similar toxicity to those separated from whole spores.

The association of toxicity with crystal protein both dissolved and undissolved indicates that the crystal substance is itself a single toxic protein. The resistance of this protein to complete dissolution with alkali does not necessarily indicate a similar resistance on the part of its toxicity. In fact toxicity appeared considerably less in pure crystals dissolved in 0.1 *N* NaOH than in those suspended in water. Studies of toxicity employing other methods of dissolving these protein crystals are in progress and will be reported separately.

The toxicity in a culture during the later stages of spore and crystal formation is more readily extracted by alkali than that of the finished crystal. This suggests that either a more soluble crystal precursor exists in the cytoplasm of the crystal-forming cells or, more probably, at the end of sporulation the crystals undergo a ripening process associated with a decrease in solubility. These findings have prompted further studies of the mode of formation of crystals and of their solubility and structure.

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STIMULATION OF GLUCOSE OXIDATION IN EXTRACTS OF BACTERIAL SPORES BY DIPICOLINIC ACID[1]

WILLIAM K. HARRELL

Abstract

The addition of pure dipicolinic acid to cell-free extracts of spores of *Bacillus cereus* var. *terminalis* results in an increase in oxygen uptake resulting from the oxidation of glucose. This stimulation, in undialyzed extracts, is proportional to the amount of DPA added up to 3 mg. per ml. of extract.

Dialysis of the extract against phosphate buffer, Tris buffer, and glycyl glycine buffer results in a decrease in glucose oxidation as compared to non-dialyzed extracts. Replacement of the DPA loss during dialysis increases the oxygen uptake to approximately the same level as obtained with non-dialyzed extracts without added DPA. The addition of Versene (EDTA) rather than DPA to dialyzed and non-dialyzed extracts also stimulates the oxidation of glucose.

Calcium reduces the enzymatic activity by approximately one-half in dialyzed extracts. This inhibition is completely reversed by replacing the DPA loss during dialysis.

The possibility is discussed that one of the functions of DPA in the spores of aerobic bacilli is the removal, by chelation, of some inhibitory metallic ion, probably calcium.

Introduction

The dormancy of bacterial endospores towards heat resistance and enzyme activity has been known for many years. However, there has been very little evidence that would indicate the regulatory mechanism for such dormancy. The unique presence of large concentrations of dipicolinic acid (DPA) in the spores of aerobic (14, 13, 3) and anaerobic (4, 6) bacilli suggests that this compound may play a special role in these cells. Also, the demonstration that this material is synthesized during the formation of the spore and released upon germination further suggests that DPA is an important constituent of these cells.

The enzymes present in intact spores of *Bacillus cereus* var. *terminalis* involved in the oxidation of glucose, can be activated, without concomitant germination, by a prolonged heat treatment (2) or by incubating the spores in low levels of germinating agents (12, 11, 10). Also, these enzymes are active in cell-free extracts of these spores (6). Although extracts from activated spores oxidize glucose as rapidly as intact, activated spores, extracts from non-activated spores contain only 25% of this activity. The release of DPA in parallel with the activation of glucose oxidation by intact spores (8) prompted the following investigation of its possible role in dormancy.

Experimental Methods

Preparation of Spore Suspensions

The spores of *B. cereus* var. *terminalis* were used throughout this work. Clean spore suspensions were prepared as described by Church *et al.* (3) and

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Contribution from the Department of Microbiology, West Virginia University Medical Center, Morgantown, West Virginia, U.S.A. This investigation was supported by research grant E-1117 from the National Institutes of Health, U.S. Public Health Service.

maintained in the lyophilized state at −20° C. until used. These suspensions contained less than 1% germinated forms. Before being used, the spore suspensions were washed twice with distilled water, suspended in 0.067 *M* phosphate buffer, pH 7.4, and heat-shocked at 60° C. for 1 hour.

Spore Extract

Cell-free extracts were prepared by suspending 2 g. of dry spores in 20 ml. of phosphate buffer and grinding in a Waring blendor at 0–4° C. for 30 minutes. Twenty grams of "Superbrite" glass beads (No. 110) were used as an abrasive. This resulted in approximately 90% breakage of the spores. After centrifugation at 10,000×*g* for 20 minutes in the cold, the supernatant was used for the studies reported here.

Manometric Experiments

Standard Warburg methods were employed for the determination of glucose oxidation by the spore extracts. The extracts were supplemented with 0.005 *M* diphosphopyridine nucleotide. Glucose, at a final concentration of 25 m*M*, was used as the substrate. All reactions were measured at 30° C. with air as the gas phase. The center well contained a fluted filter paper saturated with 0.2 ml. of 20% potassium hydroxide.

Measurement of Dipicolinic Acid

Dipicolinic acid was measured as previously reported (13, 8). The calcium salt was prepared from hydrolyzed extracts and measured at 278 mμ using a Beckman DU spectrophotometer.

Results

Effect of DPA on Dialyzed and Non-dialyzed Extracts

The addition, to the Warburg vessels, of 4 mg. of pure DPA resulted in a considerable increase in the oxygen uptake resulting from the oxidation of glucose. Results of a typical experiment are shown in Fig. 1. Using 1 ml. of extract there was approximately a twofold increase in oxygen consumption during the 60-minute test period. It should be pointed out that the undialyzed extracts contain, in addition to the pure DPA added, that which was released from the spore during breakage. The latter amounts to approximately 8.5 mg. per ml. of extract, depending on the concentration of spores initially used and the amount of breakage obtained. This stimulation is proportional to the amount of DPA added, up to 3 mg. per ml. of extract, after which there is a sharp break in the curve (Fig. 2). The DPA was not metabolized by these extracts and could be quantitatively recovered at the end of the experiment.

In order to remove the "endogenous" DPA liberated during the breakage of the spores, extracts were dialyzed overnight against 0.067 *M* phosphate buffer at 4° C. This results in a decrease in oxygen uptake as compared to the non-dialyzed extracts (Fig. 3). Replacement of the DPA loss during dialysis, i.e., 8.5 mg. per ml. of extract, increases the oxygen uptake to approximately

the same level as the non-dialyzed extracts without added DPA. The addition of more than 8.5 mg. is slightly inhibitory to the system rather than giving an additional stimulation. The reason for this is not known at this time.

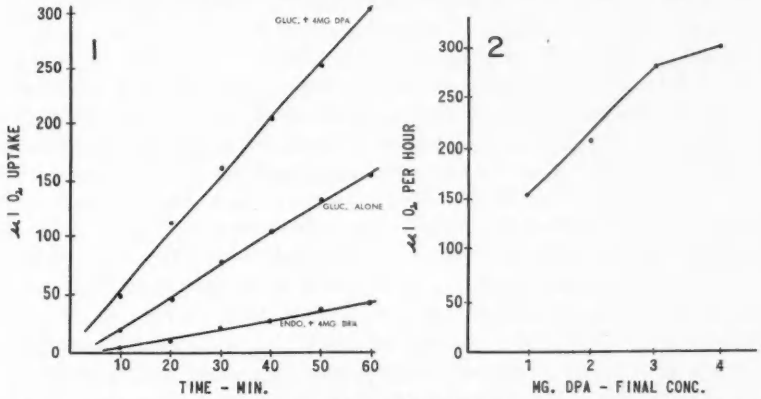

FIG. 1. Stimulation of glucose oxidation by DPA. 1 ml. of non-dialyzed spore extract obtained from 2 g. of spores heat-shocked for 60 minutes at 60° C.; 0.005 *M* DPN; 25 m*M* glucose; made up to 2 ml. with 0.067 *M* phosphate buffer; DPA added as indicated; 0.2 ml. of 20% KOH in the center well.

FIG. 2. Effect of various concentrations of DPA on glucose oxidation by non-dialyzed spore extract. Conditions same as in Fig. 1.

Effect of Ethylenediaminetetraacetic Acid (EDTA) and Metallic Ions on Glucose Oxidation

Recently Powell and Strange (15) reported that DPA strongly chelates a number of metals including calcium, copper, nickel, iron, and manganese. From this work the possibility that the stimulation reported above resulted from chelation and removal of some inhibitory metallic ion was considered.

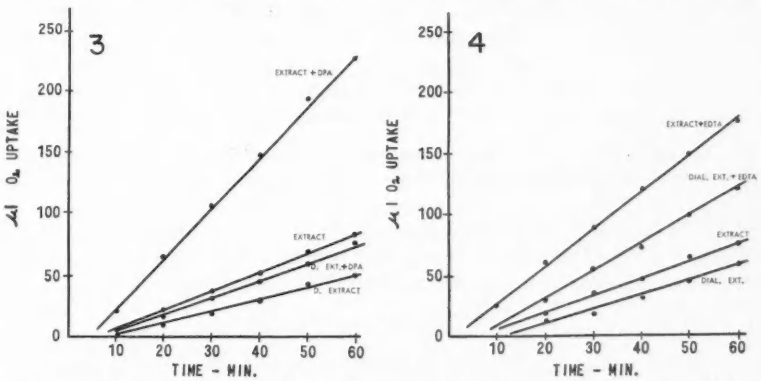

FIG. 3. Effect of DPA on dialyzed and non-dialyzed spore extracts. Conditions same as in Fig. 1; 8.5 mg. DPA added to dialyzed extracts; 3 mg. DPA added to non-dialyzed extract.

FIG. 4. Effect of ethylenediaminetetraacetic acid on dialyzed and non-dialyzed spore extracts. Conditions same as in Fig. 1; EDTA added at a final concentration of 0.006 *M*.

To test this possibility a known chelating agent, EDTA, was added to the system rather than DPA. From Fig. 4 it can be seen that EDTA at a final concentration of 0.006 *M*, stimulated the oxygen uptake in both dialyzed and non-dialyzed extracts. With dialyzed extracts the stimulation was greater when EDTA was added to the system than when DPA was added.

The higher concentration in spores of Cu^{++}, Mn^{++}, and particularly Ca^{++} (5) has led to various suggestions that they may play a role in dormancy. Since DPA is released primarily as the calcium salt during germination or after a prolonged heat treatment, this was the first of several metals tested to determine their effect upon the enzymes being studied in these extracts. The addition of calcium chloride, in a final concentration of 10^{-3} *M*, to 1 ml. of dialyzed extract reduced the activity by approximately one-half (Fig. 5). Reconstituting the DPA lost during dialysis results not only in a complete reversal of the inhibition but also a stimulation of the same magnitude as the dialyzed extract plus DPA, but without calcium added.

Because of the precipitation of calcium in the presence of phosphate buffer, these experiments were repeated using extracts prepared in Tris buffer and glycyl glycine buffer (7) with comparable results.

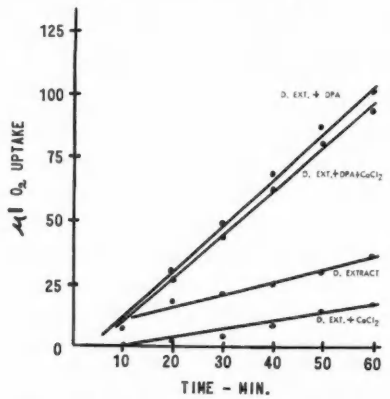

FIG. 5. Effect of calcium on dialyzed spore extract. Conditions same as Fig. 1; $CaCl_2$ added at a final concentration of 10^{-3} *M*; 8.5 mg. DPA added.

Other heavy metals tested were zinc, copper, and mercury; all caused an inhibition of glucose oxidation which was reversed by DPA. The addition of ferrous ion, on the other hand, stimulated glucose oxidation, but not to the same extent as DPA.

Discussion

The synthesis of DPA concurrent with sporulation (13), its release on germination, and its complete absence (14, 13, 8) in vegetative cells certainly imply that this material plays a unique role in the physiology of spores of

aerobic bacilli. This, together with the fact that this material constitutes upward of 15% of the dry weight of the spore, makes it imperative that the presence of DPA be explained before a complete understanding of the physiology of the spore can be obtained.

Although the function of DPA in resting spores still remains unsolved, experiments reported here indicate that at least one of its functions may be the removal of some inhibitory metallic ion during the period of activation. It is not possible to demonstrate any metabolic activity in resting spores; however, after a prolonged heat treatment or germination, glucose is oxidized at an active rate (2). This activation is accompanied by a release of DPA (8). Thus it has not been possible to activate the enzymes present in spores without the release of this material. The inhibition of glucose oxidation by the addition of calcium to the extracts and its reversal by DPA would indicate a possible mechanism for removal of the excess calcium present in the spores. This possiblity is further suggested by the observation that a known chelating agent, ethylenediaminetetraacetic acid, produces comparable results.

One of the questions that remains unanswered is why DPA stimulates glucose oxidation in the dialyzed as well as the non-dialyzed extracts. One would expect most, if not all, of the excess metals to be removed during dialysis of the extract. However, preliminary studies have shown that after dialysis approximately one third of the initial concentration of calcium remains in the extract.* Since this is apparently bound calcium, the possibility exists that this amount is sufficient to cause the lower activity obtained in the dialyzed extracts. If this is true, then the addition of DPA could conceivably remove the remaining excess calcium, which, in turn, would result in the observed stimulation.

This is not to say that calcium is not required by this system. Halvorson *et al.* (7) have demonstrated the presence of adenosine triphosphatase in extracts of spores of *B. cereus* var. *terminalis*. It has been shown that calcium is an activator of ATPase, but will inhibit respiration (9) in a manner analogous to its regulation of respiration in heart preparations (16). The mandatory coupling of electron transport to oxidative phosphorylation in other systems (1) and the sensitivity of ATPase to certain heavy metals (9) suggest at least one site for such inhibition in spores.

From the results of these studies, it would appear that at least one of the functions of DPA in the activated or germinating spore is the removal of some excess metallic ion, possibly calcium, which otherwise would inhibit the enzymatic activity of the resting spore.

Acknowledgments

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*Harrell, W. K. Unpublished results.

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THE ANTIALGAL ACTIVITY OF ACTI-DIONE[1]

ALFONS ZEHNDER[2] AND ELWYN O. HUGHES[3]

Abstract

Acti-dione inhibits the growth of Chlorophyceae, Xanthophyceae, and Bacillariophyceae at concentrations of 50 p.p.m. or less, but much higher concentrations have no inhibiting effect on the development of Myxophyceae. At concentrations of 50 to 100 p.p.m., it has been successfully employed in the isolation of unialgal cultures of two species of Myxophyceae. *Haematococcus lacustris*, a species of Chlorophyceae, has the lowest tolerance to Acti-dione of any microorganism yet found. Acti-dione interferes with cell division of this species at very low concentrations (0.016 to 0.032 p.p.m.), but much higher concentrations are required to inhibit motility. The effect of Acti-dione may be algistatic or algicidal depending upon the concentration used, the duration of the exposure, the species concerned, and the cell density.

Introduction

Pure cultures are indispensable for studies of the physiological characteristics of microorganisms. Cultures of Myxophyceae (blue-green algae), especially of Chroococcales, are difficult to purify because the gelatinous sheaths surrounding the single cells or colonies of many species frequently trap contaminating microorganisms and these are difficult to remove by mechanical means. This situation sometimes makes it difficult even to obtain unialgal cultures, because small-celled species of Chlorophyceae (green algae) or Bacillariophyceae (diatoms) may be among the contaminants inhabiting the sheaths. Unialgal cultures can sometimes be obtained by repeated subculturing on a medium favoring the growth of a desired species. This procedure is generally very time-consuming, however, and when tried with the Myxophyceae often ends in failure because suitable enrichment media are not available.

Acti-dione is an antibiotic produced by certain strains of *Streptomyces griseus* (3). Its production, assay, antibiotic activity, structure, and chemistry have all been investigated (6, 9). It is known to be active against many yeasts and fungi, but to be tolerated in high concentrations by most bacteria. In tests of the antialgal properties of 76 different chemical compounds, Palmer and Maloney (7) found that Acti-dione suppressed the development of two species of Chlorophyceae and two species of Bacillariophyceae but not that of two species of Myxophyceae. This suggested the possibility that Acti-dione might be toxic to many, if not all, Chlorophyceae and Bacillariophyceae but non-toxic in relatively high concentrations to most Myxophyceae. The experiments to be described in this paper were

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Issued as N.R.C. No. 4811.
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undertaken primarily to test this hypothesis and show whether or not Acti-dione could be used to eliminate undesired algal contaminants from cultures of Myxophyceae. The mode of action of Acti-dione was also investigated. This paper amplifies and extends what has already appeared in abstract form (11).

Materials and Methods

Species of algae to be tested were obtained from the culture collections of the University of Indiana, Bloomington (B); Cambridge University, England (C); National Research Council, Ottawa, Ontario (NRC); Prof. Robert Emerson, University of Illinois, Urbana (E); Prof. Hans Gaffron, University of Chicago (G); or from the collection of one of the authors (Z). In addition, mixed samples of algae from various habitats were tested.

Since the nutritional requirements of the species tested were not the same, five different culture solutions were employed (Table I). In some cases these solutions were solidified with 1.5% agar (Difco). Cotton-stoppered test tubes or Erlenmeyer flasks were employed as culture vessels. All solutions were autoclaved before use.

Acti-dione was obtained from the Upjohn Company, (Kalamazoo, Mich., U.S.A.). Tared samples of the antibiotic were sterilized by 30-minute ultraviolet irradiation (General Electric 30-w. germicidal tube, distance 50

TABLE I

COMPOSITION OF THE DIFFERENT CULTURE SOLUTIONS USED IN THIS STUDY

	Solution				
	A,* mg.	F,† mg.	J,‡ mg.	K,§ mg.	10,‖ mg.
$NaNO_3$	200	124	–	–	372
KNO_3	–	–	–	333	–
NH_4NO_3	–	–	100	–	–
NH_4Cl	10	–	–	–	–
$K_2HPO_4.3\ H_2O$	65	13	100	67	39
$MgSO_4.7\ H_2O$	50	25	100	33	50
$CaCl_2.2\ H_2O$	13	36	10	–	36
$Ca(NO_3)_2.4\ H_2O$	–	–	–	48	–
Na_2CO_3	–	20	–	–	20
$Na_2SiO_3.9\ H_2O$	–	58	–	–	58
Fe-citrate	3	3	5	2	6
Citric acid	3	3	5	2	6
Gaffron's minor element solution¶	0.04 ml.	0.04 ml.	0.2 ml.	0.05 ml.	0.08 ml.
Soil extract**	–	40 ml.	–	3 ml.	–
Demineralized water	1000 ml.	960 ml.	1000 ml.	997 ml.	1000 ml.

*Modification of Allen's solution No. 3 (1).
†Modification of Fitzgerald's solution (2).
‡Modification of a solution used by Jacobsen (5).
§Modification of Knop's solution.
‖Used by us temporarily to culture *Microcystis aeruginosa*.
¶Composition given in (4).
**Prepared by boiling 100 cc. of carbonated soil with 500 ml. of distilled water for 30 minutes. The supernatant was decanted and filtered. Carbonated soil = powdered $CaCO_3$. 20 cc. and powdered $Ca(OH)_2$, 10 cc., mixed thoroughly with 1670 cc. dry soil.

cm.) and then dissolved in steriie water to give 10-fold concentrated stock solutions. One milliliter of Acti-dione stock solution was added to each 9 ml. of autoclaved culture solution. Controls received 1 ml. of sterile water instead. The Acti-dione was added to agar slants just before jelling occurred. Cultures were inoculated with either a pipette or a platinum loop. Unless otherwise stated, the cultures were incubated at room temperature (about 25° C.) and continuously illuminated by fluorescent light (100–250 ft-c.) for 3 weeks.

The experiments involving different species of algae or natural collections were evaluated by macroscopic and/or microscopic comparison of the treated and control cultures. Cell counts were made in a number of experiments. In others, toxicity towards certain species or the elimination of contaminants was assessed by subculturing in solutions containing no Acti-dione.

TABLE II

MYXOPHYCEAE THAT TOLERATED ACTI-DIONE IN CONCENTRATIONS UP TO AND INCLUDING 200 P.P.M.

Species	Source* and strain No.	Culture solution
Chroococcales		
Aphanocapsa sp.	Z–36	K agar
Microcystis aeruginosa	NRC–1	10
Gloeothece rupestris	Z–37	K agar
Coelosphaerium kützingianum	C–1414–1	F
Hormogonales		
Oscillatoria tenuis	C–1459–4	A
Phormidium foveolarum	C–1462–1	A
Lyngbya sp.	C–1446–2	A
Anabaena cylindrica	C–1403–2	A
Tolypothrix tenuis	C–1482–3	A
Tolypothrix sp.	Z–38	K agar
Gloeotrichia echinulata	C–1432–1	F

*Indicated by code letters as given in text.

Results

Myxophyceae

Eleven species of Myxophyceae were grown in appropriate culture solutions containing 0, 1, 5, 20, 50, 100, or 200 p.p.m. of Acti-dione. Three of the 11 species were aerophytic and so were grown on agar slants instead of in solution. After 3 weeks, none of the 11 species had been inhibited by Acti-dione to a detectable degree (Table II). *Gloeothece rupestris* (Lyngb.) Bornet was allowed to grow for about two months. After this time, cultures of this species containing 100 and 200 p.p.m. of Acti-dione appeared slightly less vigorous than the controls. Cultures containing 50 p.p.m., however, were not affected.

Chlorophyceae, Xanthophyceae, Bacillariophyceae

Tests with 10 species of Chlorophyceae and with one species each of Xanthophyceae and of Bacillariophyceae gave entirely different results (Table III). As little as 1.0 p.p.m. of Acti-dione completely inhibited the growth of five species. A second group tolerated 1 and 5 but not 20 p.p.m.,

TABLE III

CONCENTRATIONS OF ACTI-DIONE COMPLETELY INHIBITING GROWTH OF CHLOROPHYCEAE, XANTHOPHYCEAE, AND BACILLARIOPHYCEAE

Acti-dione, p.p.m.	Species	Taxonomy*	Source† and strain No.	Culture solution
1	*Haematococcus lacustris*	C, V	Z–102	J
	Ankistrodesmus falcatus	C, Ch	B–188	K
	Scenedesmus obliquus	C, Ch	G–D3	K
	Raphidonema longiseta	C, U	B–339	K
	Tribonema aequale	H, X	B–50	K
20	*Chlorella pyrenoidosa*	C, Ch	E	A and K
	Hormidium subtile	C, U	Z–190	K
	Navicula minima	H, B	B–391	K
50	*Chlamydomonas agloeformis*	C, V	B–231	K
	Coccomyxa elongata	C, Ch	B–267	K
	Chlorococcum minutum	C, Ch	B–117	K
	Stichococcus bacillaris	C, U	B–314	K

*C = division Chlorophyta, H = division Chrysophyta, Ch = Chlorococcales, U = Ulotrichales, V = Volvocales, B = Bacillariophyceae, X = Xanthophyceae.
†Indicated by code letters as given in text.

and a third group was completely inhibited only with 50 p.p.m. of Acti-dione. No relation between Acti-dione sensitivity and taxonomic position within the Chlorophyceae could be shown: *Haematococcus lacustris* (Girod.) Rostaf and *Chlamydomonas agloeformis* Pascher (both belonging to the Volvocales) had quite different sensitivities. The former was inhibited by 1.0 p.p.m. or even 0.03 p.p.m. (as shown in a further experiment), while *Chlamydomonas* tolerated more than 20 p.p.m. Similar differences were observed between *Raphidonema longiseta* Vischer and *Stichococcus bacillaris* Nageli (both belonging to the Ulotrichales) and among the five members of the Chlorococcales (*Ankistrodesmus falcatus* (Corda) Ralfs, *Scenedesmus obliquus* (Turp.) Kg., *Chlorella pyrenoidosa* Chick, *Coccomyxa elongata* Chodat et Jaag, and *Chlorococcum minutum* Starr. The behavior of *Tribonema aequale* Pascher and *Navicula minima* Grun. demonstrated the sensitivity of representatives of the Xanthophyceae and Bacillariophyceae to Acti-dione. *Chlorella* showed the same sensitivity when cultured and tested in either modified Knop's solution or modified Allen's solution No. 3.

Mixed Cultures

Mixed cultures consisting of eight representatives of the Myxophyceae and nine representatives of the Chlorophyceae were tested in three different solutions (A, F, K) with Acti-dione in concentrations ranging from 0 to 100 p.p.m. The same species were used as in the previous experiments (Tables II and III), but *Aphanocapsa* sp., *Gloeothece rupestris*, *Tolypothrix* sp., and *Coccomyxa elongata* were omitted. After 27 days, both Chlorophyceae and Myxophyceae had developed in the control cultures while in the 100 p.p.m. Acti-dione cultures only Myxophyceae had grown.

It was not altogether surprising that, even in the control cultures, the various species developed differently. In solution K, for example, the Chlorophyceae outnumbered the Myxophyceae while in solution F the Chlorophyceae and the Myxophyceae grew equally well. In solution A, however, the growth of the Myxophyceae was distinctly favored. *Raphidonema longiseta* Vischer and *Gloeotrichia echinulata* (J. E. Smith) P. Richter did not develop to a detectable degree in any culture.

Natural Collections

In a further series of experiments, six algal samples collected from various natural habitats, each containing a mixture of species, were cultivated in media containing Acti-dione. The natural habitats that were sampled included planktonic, aerophytic, soil, and rock communities. The effects of Acti-dione were evaluated after 4 to 7 weeks, depending on the growth rate.

The first assay was made with a sample of a waterbloom collected in Little Rideau Lake on June 6, 1955. The following species were observed or identified (8):

Myxophyceae:
- *Anabaena flos-aquae* (Lyngb.) De Brebisson (predominant)
- *A. circinalis* Rabenhorst
- *A. spiroides* Klebahn
- *Gloeotrichia echinulata* (J. E. Smith) P. Richter
- *Microcystis aeruginosa* Kütz. emend. Elenkin
- *Lyngbya Birgei* G. M. Smith

Bacillariophyceae:
- *Asterionella formosa* Hassall
- Some unidentified species

Chlorophyceae:
- Some unidentified representatives of the Chlorococcales

Cultures were prepared in Erlenmeyer flasks. Three solutions (A, F, K) and five Acti-dione concentrations (0, 1, 5, 20, and 50 p.p.m.) were tested. Chlorophyceae, which were very rare in the original bloom, developed in all control cultures (Table IV). They were predominant in solutions F and K,

TABLE IV

CLASSES* OF ALGAE FROM A WATERBLOOM STILL GROWING AFTER 3 WEEKS IN THREE DIFFERENT CULTURE SOLUTIONS CONTAINING VARIOUS CONCENTRATIONS OF ACTIDIONE

Acti-dione, p.p.m.	Culture solution		
	A	F	K
0	C, B, M	C, B, M	C, B, M
1	C, B, M	C, B, M	C, B, M
5	C, M	C, M	C, M
20	M	M	C, M
50	M	M	M

*C = Chlorophyceae; B = Bacillariophyceae; M = Myxophyceae.

but less abundant in solution A. Some Bacillariophyceae were growing very actively, especially in solution F. The number of Chlorophyceae and Bacillariophyceae—both number of species and number of individuals—decreased with increasing Acti-dione concentration. No Bacillariophyceae were present in cultures with 5 p.p.m. of Acti-dione. Chlorophyceae disappeared from culture solutions A or F containing 20 or 50 p.p.m. of Acti-dione, and from culture solution K containing 50 p.p.m. of the antibiotic, leaving only the Myxophyceae to develop. The various *Anabaena* species predominant in the natural bloom did not develop in vitro. *Microcystis* and *Lyngbya Birgei* grew to some extent, but representatives of the genera *Phormidium*, *Oscillatoria*, and *Lyngbya*, which could hardly be detected in the inoculum, became dominant.

Wet moss from the shore of Black Lake, Gatineau National Park, Que., was the source of the algae for a second test. A moss pad that had been partly submerged in the lake water was squeezed out by hand. Small samples of the expressed liquid containing various species of algae were inoculated on agar slants of solutions A and K with and without Acti-dione. In the control tubes, Chlorophyceae greatly predominated. (*Gloeocystis*, *Palmella*, *Oocystis*, *Ankistrodesmus*, and *Scenedesmus* were identified.) Several species of Bacillariophyceae developed to a considerable degree but were more abundant on agar A than on agar K. Only a few Myxophyceae grew to any extent. In the cultures with as little as 1.0 p.p.m. of Acti-dione, however, no Bacillariophyceae were found, the Chlorophyceae were considerably inhibited, and the Myxophyceae were abundant. With 20 and 50 p.p.m. of Acti-dione, only Myxophyceae could be detected (*Anabaena*, *Aphanocapsa*, *Dactylococcopsis*, *Nostoc*, *Oscillatoria*, *Stigonema*, and *Synechococcus* were identified.)

Similar results were obtained with algae from four other habitats. The results of all these tests are summarized in Table V to show that regardless of the culture solution, 50 p.p.m. of Acti-dione was sufficient to completely suppress the development of the Chlorophyceae and Bacillariophyceae but not the Myxophyceae.

TABLE V

CLASSES* OF ALGAE FROM VARIOUS HABITATS STILL GROWING AFTER 3 WEEKS IN CULTURE SOLUTIONS WITH AND WITHOUT 50 P.P.M. ACTI-DIONE

Inoculum		Classes of algae present after 3 weeks growth	
Source	Classes of algae present	Without Acti-dione	With Acti-dione
Waterbloom	M>C, B	C, B, M	M
Wet moss	C, B, M	C>B>M	M
Moist soil	C, M	C, M	M
Irrigated rock	B, M	M>C, B	M
Damp rock	C, M	C>M	M
Greenhouse wall	M>C	C>M	M

*C = Chlorophyceae; B = Bacillariophyceae; M = Myxophyceae.

Acti-dione has been successfully employed to obtain a unialgal culture of *Tolypothrix byssoidea* (Berkeley) Kirchner from one that had previously been contaminated with *Stichococcus bacillaris* Naeg. and *Hormidium subtile* Heering. The second subculture on agar containing 50 p.p.m. of Acti-dione proved to be free from green algal contaminants. Likewise, a culture of *Microcystis aeruginosa* that was heavily contaminated with a species belonging to the Polyblepharidaceae was purified by the use of 100 p.p.m. of Acti-dione. In this case, mechanical purification probably would have been especially tedious because all the *Microcystis* colonies contained cells of the contaminant in their gelatinous sheaths (further details are given in the next section).

Effects on Cell Division and Motility

To find out more about the effects of Acti-dione on Chlorophyceae, a number of experiments were carried out with *Haematococcus lacustris* Z–102 (a member of the Volvocales (10)). This species was chosen because it is very sensitive to Acti-dione (Table III) and its life cycle includes various stages (e.g., zoospores, microzoospores, aplanospores), which, it was felt, might respond to the antibiotic in different ways. About 5000 zoospores of *H. lacustris* were inoculated in each of a series of test tubes containing 10 ml. of solution J to which Acti-dione in concentrations ranging from 0.001 to 1.0 p.p.m. had been added. After 16 days of incubation, the algae had developed to a considerable degree only in cultures with less than 0.016 p.p.m. of Acti-dione (Fig. 1). In cultures with 0.032 p.p.m. or more of Acti-dione, only the inoculated zoospores were present. They were motionless and had settled to the bottom of the tubes. Many cells appeared to have partially decomposed. In the 0.016 p.p.m. cultures, zoospores were still swimming around and the number of cells had increased by a factor of six. In the 0.008 and 0.004 p.p.m. cultures, the number of cells had increased by factors of approximately 150 and 340, respectively. With 0.002 and 0.001 p.p.m. of Acti-dione

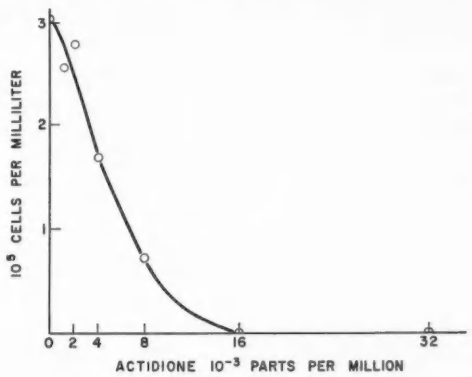

FIG. 1. Effect of Acti-dione concentration on the growth of *H. lacustris* in culture solution J (16-day cultures inoculated to give 500 cells per ml.).

there was little or no inhibition of zoospore reproduction. When cells of the "palmella-state" (10), instead of zoospores, were tested with the antibiotic the formation of zoospores was inhibited by 0.016 p.p.m., but took place within a few days in lower concentrations.

In a further experiment, Acti-dione in concentrations from 0.004 to 256 p.p.m. was added to heavy suspensions of zoospores of *H. lacustris* (about 250,000 cells per ml.). The motility was not immediately affected by the three highest concentrations. After 2 days, most cells were still moving normally. After 3 days, however, all zoospores were motionless and had settled to the bottom of the tubes (Table VI). With 4 and 1 p.p.m., it took 4 days to immobilize all zoospores. Lower concentrations produced no detectable effect on motility even after exposure for 17 days.

Cultures of *H. lacustris* grown for 2 weeks on solution J (at which time the density was approximately 250,000 cells per ml.) were treated with 1.0, 0.25, 0.0625, or 0.015 p.p.m. of Acti-dione. After 24 days, only motionless zoospores and resting cells were present, most of which had settled to the bottom of the tubes. Portions of the sediment were transferred by loop from each Acti-dione culture to flasks containing 25 ml. of solution J without Acti-dione. After 6 weeks, zoospores were abundant in the subcultures derived from the cultures containing 0.25 p.p.m. or less of Acti-dione. At these concentrations, Acti-dione was apparently algistatic. A concentration of 1.0 p.p.m. of Acti-dione, however, was apparently algicidal for the subcultures failed to develop. Rough calculations showed that the traces of Acti-dione transferred to the subcultures with the cells could not have been responsible for the lack of growth.

That Acti-dione may be algicidal under certain conditions was confirmed in another experiment involving a different representative of the Volvocales. As already mentioned, a culture of *Microcystis aeruginosa* was isolated

TABLE VI

TIME REQUIRED BY DIFFERENT CONCENTRATIONS OF ACTI-DIONE TO IMMOBILIZE ALL ZOOSPORES IN VIGOROUS CULTURES OF *H. lacustris*

Acti-dione, p.p.m.	Time, days
256, 64, 16	3
4, 1	4
0.25	7
0.0625, 0.016, 0.004, 0	>17

from a waterbloom and was found to be heavily contaminated by a species belonging to the Polyblepharidaceae. The contaminant cells were moving around very rapidly. In addition, many were either sticking to the *Microcystis* colonies or were actually trapped in their sheaths. Two days after the addition of 100 p.p.m. of Acti-dione to this culture some of the contaminant cells were still moving, but after 4 days all cells were motionless and many appeared to be partially decomposed. To see if the entrapped contaminants were dead, five *Microcystis* colonies were washed in four successive changes of sterile culture solution. The washed colonies, still containing contaminants in their sheaths, were transferred to flasks containing fresh solution No. 10. Control transfers were made with washed colonies from the untreated culture. After 6 weeks, no Polyblepharidaceae had developed in the subcultures inoculated with treated *Microcystis* colonies. In the controls, Polyblepharidaceae were abundant. It was concluded, therefore, that treatment for 4 days with 100 p.p.m. of Acti-dione had killed the contaminants.

Discussion

The work of Palmer and Maloney (7) has been extended by the present investigation to show that Acti-dione is an antibiotic to which the Chlorophyceae, Xanthophyceae, and Bacillariophyceae, but not the Myxophyceae are sensitive. Previous work by Whiffen (9) and Ford and Leach (3) has shown that Acti-dione is active against many but not all yeasts, active against many fungi, but inactive against bacteria. Among the yeasts there are species which are very sensitive to Acti-dione (e.g., *Saccharomyces pastorianus* and *S. cerevisiae*) while others tolerate more than 1000 p.p.m. (e.g., *S. lactis*). Among the Chlorophyceae, no species was found to tolerate more than 50 p.p.m., but it is quite possible that such forms exist and could be detected in more extensive studies. *Haematococcus lacustris* seems to be the most sensitive microorganism so far discovered. It is more sensitive than *Saccharomyces pastorianus*, which has hitherto been used as a test organism for Acti-dione (9).

The experiments with *Haematococcus lacustris* throw some light on the mode of action of Acti-dione. The results of the experiments with zoospores point to a blocking of cell division (Fig. 1). The formation of zoospores from "palmella-state" cells was completely inhibited by similar concentrations. These effects occur at lower concentrations than those causing loss of motility (Table VI). The behavior of *Saccharomyces cerevisiae* in a medium containing Acti-dione provides additional evidence that this antibiotic interferes somehow with cell division for a striking number of cells show abnormal division states.*

Earlier work of Whiffen (9) with *Saccharomyces pastorianus* as a test organism revealed the efficacy of Acti-dione to be a function of the cell density

*Personal communication from Dr. Zähner, Department of Special Botany, Swiss Federal Institute of Technology, Zurich, Switzerland.

of the microorganisms exposed to the antibiotic. Although no special experiments were carried out, the observation of Whiffen was indirectly confirmed with *H. lacustris*. When 500 zoospores per ml. were exposed to 0.032 p.p.m. of Acti-dione (Fig. 1), all cells were motionless after 16 days. With 250,000 zoospores per ml., however, 0.0625 p.p.m. of Acti-dione had no detectable effect on the motility of the cells (Table VI). Whether Acti-dione is algicidal or algistatic to Chlorophyceae depends upon the concentration of the antibiotic used, the duration of the exposure, the species in question, and the cell density.

The results obtained with *Navicula minima* and *Tribonema aequale* (Table III) and with natural collections (Table V) indicate that some, if not all, Bacillariophyceae and Xanthophyceae, as well as Chlorophyceae are sensitive to Acti-dione. The successful use of Acti-dione in the isolation of unialgal cultures of *Tolypothrix bysoidea* and *Microcystis aeruginosa* indicates that the addition of 50 to 100 p.p.m. of the antibiotic to the initial culture solution is likely to be of considerable help to phycologists attempting to isolate unialgal cultures of other Myxophyceae.

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SOME BIOLOGICAL PROPERTIES OF BCG AFTER THE EXTRACTION OF LIPIDS: THE POWER TO IRRITATE TISSUES, THE ABILITY TO PREPARE FOR AND TO EVOKE BAIL'S PHENOMENON, AND THE POWER TO SENSITIZE TO TUBERCULIN[1,2]

V. Portelance and A. Frappier

Abstract

Mycobacterium tuberculosis var. *bovis* BCG was treated with lipid solvents according to Macheboeuf's method and five progressively more fully extracted bacillary residues were obtained. Electron-microscope examination, together with the biological properties studied, showed that a major transformation was brought about in the bacillary residues by methanol extraction. The biological properties of the residues were studied quantitatively in guinea pigs. The power to irritate tissues (i.e., to produce tuberculous-like lesions in tissues), the ability to prepare for and to evoke Bail's phenomenon (i.e., reaction to the intraperitoneal introduction of bacillary bodies in a sensitized guinea pig characterized by an immediate leucocytic response and death within a few hours), and the power to sensitize to tuberculin were directly proportional to the lipid contents of the bacillary bodies and decreased as the extraction progressed. The residues with the lower lipid contents were less irritant, less sensitizing, and less reactive. However, these properties never completely disappeared. The role of the lipids as related to the above properties of mycobacteria was thus indirectly established. With the doses and the routes of injection used, no obvious toxic effects in normal guinea pig were observed.

Introduction

It seems logical to believe that the ideal antigen for prophylactic use against tuberculosis is one that would stimulate specific resistance without irritating the tissues or producing tuberculous-like lesions and without specifically sensitizing the individual or causing severe reactions in already sensitized individuals. If such a vaccine could be prepared without impairing its immunizing properties, it could be administered in proper doses as a prophylactic agent. It is well known that large doses of BCG vaccine cannot be injected parenterally because of the local reactions produced in normal individuals or of the local, focal, and general reactions which they can initiate in sensitized individuals. Equally impractical might be the administration of large doses of killed vaccines because whenever the amount of antigen injected exceeds a certain level, these untoward effects increase (15). Yet, vaccination with small doses of whole killed bacterial bodies yields only a weak and transient immunity. This can be enhanced by injecting them in association with adjuvants, but unfortunately, this procedure also increases the so-called toxicity of the bacterial cells.

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Contribution from the Institute of Microbiology and Hygiene and the School of Hygiene of the University of Montreal, Montreal, Quebec. This research was aided in part by a grant from the Ministry of Health of the Province of Quebec (Federal–Provincial Health Research Grants).
[2]Third communication on *Studies on Mycobacteria*. For the second one, see (8).

In both cases, lipid fractions are taking part in the undesirable phenomena (6). The local reactions produced by sub- or intra-cutaneous injections of large doses of BCG may be ascribed to the irritating action of the acetone-soluble fat which is, as established by Smithburn and Sabin (12), the most irritating of all the lipid fractions thus far isolated from mycobacteria. On the other hand, Weiss and Dubos (15) have shown that the inefficacy for immunizing mice of large doses of killed bacterial bodies may be traced to the toxicity of the bacterial suspension. In fact, Spitznagel and Dubos (13) isolated a monochlorobenzene-soluble lipid fraction possessing primary toxicity (0.59 mg. in 0.5 ml. bayol-F via the intraperitoneal route in mice). Recently, Bloch *et al.* (2) have shown that this toxic lipid fraction is a mixture of inactive glycerides and cord factor (trehalose-6,6′-dimycolate), the latter being the only toxic component of the mixture. Cord factor had previously been recognized by the latter authors as the only toxic lipid in mycobacteria.

It therefore stands to reason that the lipids are an impediment to the injection of large doses of vaccine, whether alive or killed. Consequently, it seemed of interest to study the biological properties of mycobacteria extracted with lipid solvents to discover to which extent the bacillary residues retain the biological properties of the whole bacilli. Publications on this subject are scarce and scattered at random in the literature. In 1936, Macheboeuf and Dieryck (3) reported that rabbits could be immunized against experimental tuberculous infections by vaccination with bacillary residues obtained after treatment of tubercle bacilli with acetone and ether. Studying the cellular reactions to extracted tubercle bacilli, Sabin and Joyner (10) have shown that the extraction of tubercle bacilli with neutral organic solvents yields bacillary residues giving cellular reactions similar to those known to characterize the reaction to heat-killed tubercle bacilli, while treatment with acid organic solvents gave relatively inert material. In a systematic study of the biological properties of the components of the tubercle bacillus, Raffel (9) concluded that lipid-extracted bacilli (ether–alcohol- and chloroform-extracted unkilled virulent bacilli), even when injected in large doses (10 injections of 10 mg.), did not initiate a "tuberculin type" of hypersensitiveness nor an acquired resistance in guinea pigs. Contrary to these results, Smith and Kubica (11) have recently succeeded in immunizing guinea pigs. These became tuberculin-positive with as little as 0.05 mg. of the bacillary residues remaining after neutral solvent extraction and suspended in adjuvant. A protective activity for mice of cells killed with phenol, extracted with chloroform and monochlorobenzene, and suspended in adjuvant was also established by Weiss and Dubos (14), who showed that the toxicity of these bacillary residues varied with the strains used for their preparation. These latter authors have also brought forth evidence suggesting that toxicity and protective activity were independent of each other and were the manifestations of different bacillary constituents.

The discrepancy encountered in the literature concerning the biological properties of lipid-extracted tubercle bacilli is undoubtedly due to the different experimental conditions used by the various authors. However, our review of the literature suggests that no attempt has ever been made to compare the biological properties of the bacillary residues with those of the bacilli prior to extraction by a quantitative technique. It therefore seemed of interest to study such a comparison in order to discover to which extent the bacillary residues retain the biological properties of the whole bacilli. The extraction procedure used was one that could give a series of bacillary residues with decreasing lipid content.

The present paper reports on the power of these residues to irritate tissues, to prepare for and to evoke Bail's phenomenon, and to sensitize to tuberculin. Their ability to induce acquired resistance will be reported in a forthcoming paper.

Materials and Methods

Culture and Harvesting of the Bacteria

One hundred and twenty-five 250-ml. culture flasks each containing 150 ml. of Sauton's medium were seeded with *Mycobacterium tuberculosis* var. *bovis* BCG and incubated at 38° C. for 7 days. After incubation, the cultures were combined and thoroughly mixed. The homogeneous bacterial mass was then transferred to a Büchner funnel, washed free of glycerol with water, dried in air for ½ hour, and weighed between blotting papers (semidry weight).

Extraction of the Lipids

In order to obtain a wider spectrum of bacillary residues containing progressively less lipid, these were extracted by the method of Macheboeuf (4) in a modified Soxhlet extraction apparatus as described by Macheboeuf and Fethke (5). The technique may be summarized as follows. The live air-dried bacilli (semidry weight, 68.5 g.)were placed in 450 ml. of dry acetone and the mixture was kept at 4° C. for 5 days with occasional stirring. The bacillary mass was then filtered on a Büchner funnel and this treatment was repeated once before the bacilli were finally transferred to extraction thimbles adapted with fused-in fritted glass filters. The thimbles were then placed in an extraction apparatus and the bacilli extracted with a series of three organic solvents (acetone, ether, and methanol) for a period of 25 consecutive days respectively. While being extracted, the bacilli were constantly kept at +5° C. and under vacuum. After each of these treatments, the bacillary residues were lyophilized to remove any trace of the solvent and a sample for the biological tests was stored at +5° C. under vacuum; the remainder was extracted with the next solvent. After this first series of cold extractions, each thimble was transferred to a Kumagawa apparatus for a hot extraction with a second series of solvents, ethanol and benzene. Again the bacillary residues were lyophilized and samples were kept for biological studies.

Determination of Doses for Quantitative Purposes

The use of comparable doses was made possible by taking the relation of the weight of lyophilized residues over that of the semidry BCG and is hereafter designated by *R*.

$$R = \frac{\text{weight of the lyophilized residue}}{\text{weight of the semidry BCG}} \times 100.$$

This relation was taken individually for each residue, the quantity of the previous residue which was stored for biological tests being taken into account.

Preparation of the Suspensions for Injection

The required amount of lyophilized residue was homogenzied with a few milliliters of sterile saline in a Tenbroeck apparatus and the homogenate was diluted with saline to the desired concentration. Suspensions of bacillary residues retained their homogeneity for so short a time that they had to be prepared immediately before injection. Only a single dose was taken into the syringe at one time and between each injection the suspension was homogenized by forcing it in and out of the syringe.

Results

Extraction of the Lipids

Table I shows the results of the extraction of *Mycobacterium tuberculosis* var. *bovis* BCG together with the appropriate doses calculated for each bacillary residue so as to allow quantitative studies.

It may be seen that 32.6% of the dry weight of the bacilli were extracted with neutral organic solvents thus yielding five bacillary residues hereafter designated residue I, II, III, IV, V. As indicated in the table, the required doses varied between 21.8 for residue I to 15.3 for residue V. All the residues were acid-fast and electron-microscope examination revealed that they could be divided into two series. In the first one, including residues I and II, only intact cells or very slightly altered ones could be seen; whereas in the second series, including the last three residues, the major proportion of the cells seemed to be drained of their cytoplasmic contents and the cell walls were

TABLE I

Lipid fractions of *Mycobacterium tuberculosis* var. *bovis* BCG

Solvents	Corresponding bacillary residues	Lipid extracts, %*	*R*†
(1) Cold acetone	Residue I	9.00	21.8
(2) Cold ether	Residue II	6.51	20.7
(3) Cold methanol	Residue III	12.09	17.0
(4) Boiling ethanol	Residue IV	1.85	16.4
(5) Boiling benzene	Residue V	3.24	15.3
Total free lipids		32.69	

*As % of dry weight of bacilli.
†*R* = "Calculated equivalent doses", grams of bacillary residue in 100 g. of semidry BCG.

sometimes ruptured thus showing cellular debris scattered among bacillary "ghosts". It may thus be concluded that a major transformation in the bacillary residues was brought about by methanol extraction.

The Power to Irritate Tissues

The power of the bacillary residues to irritate tissues was determined quantitatively in normal guinea pigs by measuring their reactions to intracutaneous and intraperitoneal injections of living BCG and of bacillary residues used in carefully calculated comparable doses.

1. Intraperitoneal Injection (7)

(*a*) *Technique*

Two hundred and forty tuberculin-negative guinea pigs, half male and half female, weighing approximately 500 g., were divided into eight groups of 30 pigs each. The first group served as control and was not injected. Each guinea pig of the other groups received an intraperitoneal injection of 1 ml. of the following suspensions in saline:

Group 2: 10 mg. of semidry BCG.
Group 3: 10 mg. of semidry heat-killed BCG.
Group 4: 2.18 mg. of lyophilized residue I.
Group 5: 2.07 mg. of lyophilized residue II.
Group 6: 1.70 mg. of lyophilized residue III.
Group 7: 1.64 mg. of lyophilized residue IV.
Group 8: 1.53 mg. of lyophilized residue V.

Fourteen days later, all the guinea pigs were weighed, sacrificed, and the omentum taken and weighed as rapidly as possible.

(*b*) *Results*

The quantitative interpretation of the reactions was made possible by computing the average weight of the omentum in grams per 100 g. of body weight for each group. Any irritating power is then expressed by an increase in the average weight of the omentum of treated guinea pigs over that of untreated animals. Table II shows the results obtained with the bacillary residues.

TABLE II

THE POWER TO IRRITATE TISSUES EVALUATED BY INTRAPERITONEAL INJECTION

Groups	Doses injected,* mg.	Average weight of the omentum†	Increase of the average weight of the omentum†
(1) Control	None	0.230	
(2) BCG	10	1.017	0.787
(3) Heat-killed BCG	10	0.550	0.320
(4) Residue I	2.18	0.440	0.210
(5) Residue II	2.07	0.429	0.199
(6) Residue III	1.70	0.342	0.112
(7) Residue IV	1.64	0.328	0.098
(8) Residue V	1.53	0.254	0.024

*In 1 ml. saline.
†For groups of 30 guinea pigs, expressed in grams per 100 g. of body weight.

It may be seen in Table II that the most irritant preparation was the living BCG whereas a sharp fall was registered for the heat-killed BCG when injected in the same dosage. Still less irritant were the bacillary residues injected with comparable doses. It may also be noticed that the power of the residues to irritate tissues decreases in direct proportion to the extent of extraction of lipids, the most fully extracted, residue V, being almost non-irritant. This is most obvious between residues II and III, that is after methanol extraction. It may be remembered that electron-microscope examination had also revealed a major transformation in the bacillary residues after methanol extraction.

2. Intracutaneous Injection

(*a*) *Technique*

Seventy tuberculin-negative albino guinea pigs, half male and half female, weighing approximately 450 g., were divided into seven groups of 10 pigs each (5 males and 5 females). Each animal received an intracutaneous injection, on the depilated abdomen, of 0.1 ml. of the following suspensions in saline:

Group 1: BCG, 0.01 mg./ml.
Group 2: heat-killed BCG, 0.01 mg./ml.
Group 3: lyophilized residue I, 0.0021 mg./ml.
Group 4: lyophilized residue II, 0.0020 mg./ml.
Group 5: lyophilized residue III, 0.0017 mg./ml.
Group 6: lyophilized residue IV, 0.0016 mg./ml.
Group 7: lyophilized residue V, 0.0015 mg./ml.

Seven days later, and thereafter twice a week, the surface of the intradermal nodule, expressed in square millimeters and obtained by multiplication of the smallest by the largest diameter, was read.

(*b*) *Results*

Table III shows that the results obtained by the intracutaneous injection of bacillary residues agree with those previously reported for the intraperitoneal injection. Heat-killed organisms were less irritant than the living ones and the power of the bacillary residues to irritate tissues was indeed progressively reduced as the extraction progressed. Again a deep fall was noticed after methanol extraction.

THE ABILITY TO PREPARE FOR AND TO EVOKE BAIL'S PHENOMENON

(*a*) *Technique*

In guinea pigs highly sensitized with tubercle bacilli, Bail (1) has described a reaction to the intraperitoneal introduction of bacillary bodies characterized by an immediate leucocytic response and death within a few hours. We applied a somewhat similar but more quantitative technique, and the survival rate was the only criterion used. Only the first three bacillary residues were used, the remainder of the last two residues were kept for the tuberculin sensitizing and acquired resistance tests. Groups of seven tuberculin-negative guinea pigs (450 g.) were injected first, intravenously, with 1 ml. of the following suspensions: (1) living BCG, 20 mg./ml.; (2) heat-killed BCG, 20 mg./ml.; (3) residue I, 4.36 mg./ml.; (4) residue II, 4.14 mg./ml.

No obvious toxic effects were observed in the following 3 months, after which the animals were given a second intraperitoneal injection with 1 ml. of a 10 mg./ml. suspension of BCG or of bacillary residue. In all instances, comparable doses were used. Deaths among the pigs were recorded within 22 hours after the second injection.

Cross reactions were performed according to the scheme summarized in Table IV.

TABLE III

THE POWER TO IRRITATE TISSUES EVALUATED BY THE INTRACUTANEOUS NODULE

Days after injection	Products injected						
	Living BCG	Heat-killed BCG	Residue I	Residue II	Residue III	Residue IV	Residue V
	Surface of the nodules in mm.²*						
7	178	149	136	78	45	83	65
13	266	117	113	82	40	43	45
17	255	99	49	24	26	15	20
20	158	43	49	5	0	7	4
24	73	13	7	0	0	2	0
27	43	5	9	0	0	0	0
31	34	7	0	0	0	0	0

*Smallest diameter multiplied by the largest.

TABLE IV

THE ABILITY TO PREPARE FOR AND TO EVOKE BAIL'S PHENOMENON, USING COMPARABLE DOSES

Groups of 7 guinea pigs	First injection, 1 ml. intravenously		Second injection, 1 ml. intraperitoneally		% of death within 22 hr. after second injection
	Product	Dose, mg.	Product	Dose, mg.	
1	BCG	20.00	BCG	10.00	100.0
2	BCG	20.00	Heat-killed BCG	10.00	100.0
3	BCG	20.00	Residue I	2.18	66.6
4	Residue I	4.36	BCG	10.00	71.4
5	Residue I	4.36	Residue I	2.18	43.0
6	BCG	20.00	Residue II	2.07	100.0
7	Residue II	4.14	BCG	10.00	0
8	Residue II	4.14	Residue II	2.07	0

(*b*) *Results*

Table IV shows 100% mortality in the groups of pigs prepared for Bail's phenomenon with BCG (groups 1 and 2) and reinjected with the same organisms, whether alive or killed, thus establishing a control pattern for this reaction with BCG. The mortality observed in groups 3 and 4 was 66.6 and 71.4% respectively, thus showing that, on a quantitative basis, residue I prepared the guinea pigs for Bail's phenomenon to a lower level than did BCG and also provoked it in fewer animals. This is confirmed in group 5 where only 43%

of the pigs prepared with residue I died after a second injection of the same preparation. Results obtained in group 6 indicated that residue II could, indeed, provoke Bail's phenomenon whereas groups 7 and 8 yielded results establishing that, in these experimental conditions, residue II could not prepare the pigs for Bail's phenomenon. It may thus be concluded that, on the basis of strictly comparable doses, the progressive lipid extraction of BCG tended to lower its ability to sensitize and to react. Residue I both established and provoked Bail's phenomenon to a lower degree than did untreated organisms, whereas residue II could not prepare the animal for the phenomenon and could only provoke it in animals prepared with BCG.

To discover if the activity of residues II and III could be increased by the use of larger doses, new groups of guinea pigs were first injected intravenously with 20 mg. of those bacillary residues. No obvious toxic effects were observed, and 3 months later they were reinjected with the same doses as those primarily used. Table V shows the scheme of the experiment together with the results obtained.

Comparison of Table IV (groups 4 and 5) and Table V (groups 2 and 3) shows that the difference of activity seen between residue I and residue II, when comparative preparing doses were used, was almost suppressed if massive preparing doses of residue II were injected. However, in order to obtain approximately the same level of activity, the preparing dose of residue II used had to be five times as great as that injected for residue I. Even then, about 10% less animals died within 22 hours after the second injection, thus establishing that the cold ether extraction reduced the activity. Table V (groups 4, 5, and 6) also shows that the death rate among the groups of pigs prepared with massive doses of residue III was lower than that observed for residue II in the same experimental conditions. Hence, methanol extraction also reduced the activity of the bacillary residues.

The Power to Sensitize to Tuberculin

The power of the bacillary residues to sensitize animals to tuberculin was determined in the guinea pigs used to study Bail's phenomenon and also in those used for testing the acquired resistance.

TABLE V

The ability to prepare for and to evoke Bail's phenomenon, using massive preparing doses

Groups of 7 guinea pigs	First injection, 1 ml. intravenously		Second injection, 1 ml. intraperitoneally		% of death within 22 hr. after second injection
	Product	Dose, mg.	Product	Dose, mg.	
1	BCG	20	Residue II	2.07	83.3
2	Residue II	20	Residue II	2.07	33.3
3	Residue II	20	BCG	10.00	62.5
4	BCG	20	Residue III	1.70	57.0
5	Residue III	20	BCG	10.00	50.0
6	Residue III	20	Residue III	1.70	14.3

(*a*) *Technique*

Two series of tuberculin-negative guinea pigs were used. The first one included groups of 7 animals injected intravenously with varying doses of BCG or bacillary residues as previously described. The second series consisted of pigs immunized with varying amounts of residues and divided into five groups of 30 animals weighing approximately 400 g. The first group consisted of pigs immunized by six scarifications made on their depilated back through drops of a 13 mg./ml. suspension of residue I in saline. A second group of similar guinea pigs were immunized, by the same technique, with a 60 mg./ml. suspension of BCG and served as controls. The last three groups of pigs were first injected intraperitoneally and the next week they received a series of six scarifications followed, 5 days later, by a second series of scarifications as described below:

Group 3: heat-killed BCG. I.P., 1 ml. of a 90 mg./ml. suspension; scar., with a 180 mg./ml. suspension.

Group 4: residues I plus II (equal parts). I.P., 1 ml. of a 20 mg./ml. suspension; scar., with a 40 mg./ml. suspension.

Group 5: residues III+IV+V (equal parts). I.P., 1 ml. of a 15 mg./ml. suspension; scar., with a 30 mg./ml. suspension.

On account of the small amounts available, the residues had to be combined into two series based on the results of the electron-microscope examination and on the decrease of irritating power observed after methanol extraction.

Two months after the last injection, all the guinea pigs were tuberculin-tested with graded doses of old tuberculin administered in 0.1-ml. amounts intracutaneously on the depilated abdomen. Forty-eight hours later, the surface of the oedema was measured by multiplying the smallest by the largest diameter and only reactions of 25 sq. mm. or more were considered as positive.

TABLE VI

THE POWER OF BACILLARY RESIDUES TO SENSITIZE TO OLD TUBERCULIN. PERCENTAGE OF GUINEA PIGS WITH POSITIVE REACTIONS*

Series of guinea pigs	Animals		Product injected	Dose and route of injection	% of animals with positive reactions to O.T.†		
	Group	Number			10 mg.	1.0 mg.	0.1 mg.
I	1	7	BCG	20 mg. I.V.	100	100	25
	2	7	Residue I	4.36 mg. I.V.	100	43	0
	3	7	Residue II	4.14 mg. I.V.	33	25	0
II	1	30	Residue I	Sc. 13	22.7	—	0
	2	30	BCG	Sc. 60	100	—	37
	3	30	Heat-killed BCG	90 mg. I.P. +2 Sc. 180	85	—	—
	4	30	Residues I+II	20 mg. I.P. +2 Sc. 40	47	—	—
	5	30	Residues III+ IV+V	15 mg. I.P. +2 Sc. 30	29	—	—

*Two months after the last injection. Only reactions of 25 mm.² or more were considered as positive. Read 48 hours after injection.

†In 0.1-ml. amounts intracutaneously.

NOTE: I.V., intravenously; I.P., intraperitoneally; Sc., six scarifications with mg./ml. suspension; —, test not performed.

(*b*) *Results*

Table VI shows the scheme of the experiment together with the results obtained. It may be seen that 100% of the guinea pigs injected intravenously with 4 mg. of residue I gave positive reactions to 10 mg. O.T. whereas only 43% reacted to 1 mg. Fewer animals injected with residue II reacted to O.T. (33%), thus showing that this latter preparation sensitized the pigs to a lower level than did residue I. Both residues were less effective than BCG. Administered by a single treatment of scarifications, residue I induced positive reactions in only 22% of the animals as compared to 100% when injected intravenously, thus indicating that the former route of injection was much less effective than the latter one. Massive injections of bacillary residues gave a higher percentage of reactive animals, 47% of those in the first series (residues I+II) and 29% in the second one (residues III+IV+V) gave positive reactions to 10 mg. O.T.

However, animals of both series were less highly sensitized than those treated with heat-killed BCG.

It may thus be concluded that bacillary residues did sensitize guinea pigs to old tuberculin, but to a lower level than that reached with heat-killed or living BCG. Moreover, progressive extraction of the lipids reduced the power of the bacillary residues to sensitize to tuberculin.

Discussion

Mycobacterium tuberculosis var. *bovis* BCG was extracted according to Macheboeuf's method in order to obtain a series of bacillary residues containing progressively less lipids. Five bacillary residues were obtained and 32% of the dry weight of the bacilli extracted as lipids. Comparative doses were made possible by comparing the semidry weight of BCG with the weight of lyophilized residues. Electron-microscope examination revealed a major transformation brought about in the bacillary residues by methanol extraction. After this treatment, the cells seemed to be drained of their cytoplasmic contents and bacillary "ghosts" were frequently seen among cellular debris, whereas they were only slightly altered before methanol extraction. It thus seemed that an important cellular material was extracted by methanol.

Both the intraperitoneal and intracutaneous injections of bacillary residues showed a decrease of the power to irritate tissues as the extraction progressed, residue V being almost non-irritant. Heat-killed organisms were much less irritant than living ones and a sharp fall of activity was noticed after methanol extraction. Our results agree with those of Smithburn and Sabin (12), who reported that lipids were responsible for the irritating action of mycobacteria.

With a technique similar to that used to study Bail's phenomenon and using doses carefully adjusted for comparative purposes, it was possible to show that the ability of the bacillary residues to establish and to provoke Bail's phenomenon decreased directly with the extent of extraction, residue I being less active than living BCG, and residue II less than residue I. This result suggests that this activity may be ascribed to the lipids which have also been

shown to be responsible for the primary toxicity of the tubercle bacillus by Spitznagel and Dubos (13) and by Bloch *et al.* (2). It is then not surprising that the bacillary residues, which contained progressively less lipids, were also less able to elicit Bail's phenomenon. However, the use of massive preparing doses has shown that some activity remains in the residues and that the methanol extraction in particular reduced its specific activity. It was beyond the scope of this experiment to establish if the activity observed was truly of the delayed hypersensitivity rather than of the immediate or anaphylactic type.

Tuberculin hypersensitivity was established in guinea pigs after the intravenous injection of 4 mg. of residue I or of residue II, but the latter had a lower sensitizing power. With small doses of residue, the scarification method was less effective than the intravenous one. With massive and repeated injections of bacillary residues in the two series studied, residues I+II and residues III+IV+V gave 47% and 29% positive reaction to 10 mg. O.T. respectively. In both series, the residues were less effective than heat-killed BCG. These results are similar to those published by Smith and Kubica (11), but disagree with Raffel's conclusion that lipid-extracted bacilli (ether–alcohol- and chloroform-extracted unkilled virulent bacilli), even when injected in large doses, fail to stimulate a tuberculin type of hypersensitivity. Raffel's conclusion is all the more surprising as he himself has shown that tuberculin hypersensitivity could be induced by mixtures of extracted bacilli and wax, both preparations being inactive when injected separately. Now, it is well known that, as long as they are extracted only with neutral organic solvents, the treated bacilli retain their firmly bound lipids, the chemical composition of which has been shown by Anderson to be similar to that of the waxes. In Raffel's experiments, the presence of protein in the wax fraction was indicated "not only by the fact of sensitization to tuberculin, but in addition by the demonstration in the serum of all guinea pigs receiving the waxes of the presence of antibodies for protein". A similar combination, wax (or firmly bound lipids) plus protein, exists in the bacillary residues. Therefore, it seems logical that they should initiate a tuberculin type of hypersensitivity, though maybe only at a lower level than that following the use of heat-killed organisms.

The results of tests done to determine the specific resistance of guinea pigs immunized with several of these bacillary residues will be published in a forthcoming paper.

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NOTES

INTERCONVERSION OF BASE 10 AND BASE 2 LOGARITHMS

W. R. LOCKHART

In graphic and mathematical interpretations of bacterial growth it is usually most convenient to express cell counts or other measurements of population density on a logarithmic scale. Monod (2) has pointed out the advantages of using base 2 logarithms for such manipulations; an increase of one logarithmic unit becomes equivalent to a doubling of the population, i.e., a single generation. Finney *et al.* (1) have prepared tables of base 2 logarithms which make it quite convenient to express results in this form. It sometimes happens, however, that it is expedient to use both base 10 and base 2 logarithms for different calculations with the same set of data. Accordingly, these tables have been calculated to make possible a direct conversion of logarithms from one base to the other. Tables I and II were prepared by multiplying the indicated $\log_{10}$ values by 3.32193 ($= \log_2 10$), and Tables III and IV by multiplying the $\log_2$ values by 0.30103 ($= \log_{10} 2$).

TABLE I

CONVERSION FROM BASE 10 TO BASE 2 LOGARITHMS

$\text{Log}_{10} N$	$\text{Log}_2 N$	N
1.000	3.322	10
2.000	6.644	10^2
3.000	9.966	10^3
4.000	13.288	10^4
5.000	16.610	10^5
6.000	19.932	10^6
7.000	23.253	10^7
8.000	26.575	10^8
9.000	29.897	10^9
10.000	33.219	10^{10}
11.000	36.541	10^{11}
12.000	39.863	10^{12}

In converting from one system of logarithms to the other, the characteristic and mantissa are separated and their conversion values found in the appropriate tables. The two values are then added. For example, to convert $\log_{10} N = 8.31$ to $\log_2 N$, one would first find in Table I that the characteristic 8.00 is equivalent to 26.575 in base 2 logarithms. In Table II, the mantissa 0.31 is found to be equivalent to 1.030. Adding the values for characteristic and mantissa, we find that

$$\log_{10} N = 8.00 + 0.31 = 8.31$$
$$\log_2 N = 26.575 + 1.030 = 27.605.$$

Can. J. Microbiol. 4 (1958).

Similarly, from Tables III and IV, if

$$\log_2 N = 23.65 = 23.00 + 0.65,$$

then

$$\log_{10} N = 7.120 = 6.924 + 0.196.$$

Essentially the tables are to be used to two decimal places; the third place appears in the entries to permit more accurate rounding off after addition of the separate values for characteristic and mantissa. Inclusion of a third decimal place would increase the size of Tables II and IV by a factor of 10

TABLE II

CONVERSION FROM BASE 10 TO BASE 2 LOGARITHMS

	$\text{Log}_2 N$									
$\text{Log}_{10} N$	0	1	2	3	4	5	6	7	8	9
0.00	0.000	0.033	0.066	0.010	0.133	0.166	0.199	0.233	0.266	0.299
0.10	0.332	0.365	0.399	0.432	0.465	0.498	0.532	0.565	0.598	0.631
0.20	0.664	0.698	0.731	0.764	0.797	0.830	0.864	0.897	0.930	0.963
0.30	0.997	1.030	1.063	1.096	1.129	1.163	1.196	1.229	1.262	1.296
0.40	1.329	1.362	1.395	1.428	1.462	1.495	1.528	1.561	1.595	1.628
0.50	1.661	1.694	1.727	1.761	1.794	1.827	1.860	1.894	1.927	1.960
0.60	1.993	2.026	2.060	2.093	2.126	2.159	2.192	2.226	2.259	2.292
0.70	2.325	2.359	2.392	2.425	2.458	2.491	2.525	2.558	2.591	2.624
0.80	2.658	2.691	2.724	2.757	2.790	2.824	2.857	2.890	2.923	2.957
0.90	2.990	3.023	3.056	3.089	3.123	3.156	3.189	3.222	3.255	3.289

TABLE III

CONVERSION FROM BASE 2 TO BASE 10 LOGARITHMS

$\text{Log}_2 N$	$\text{Log}_{10} N$	N	$\text{Log}_2 N$	$\text{Log}_{10} N$	N
1.000	0.301	2.000	21.000	6.322	2.097×10^{6}
2	0.602	4.000	22	6.623	4.194×10^{6}
3	0.903	8.000	23	6.924	8.389×10^{6}
4	1.204	1.600×10	24	7.225	1.678×10^{7}
5	1.505	3.200×10	25	7.526	3.355×10^{7}
6.000	1.806	6.400×10	26.000	7.827	6.711×10^{7}
7	2.107	1.280×10^{2}	27	8.128	1.342×10^{8}
8	2.408	2.560×10^{2}	28	8.429	2.684×10^{8}
9	2.709	5.120×10^{2}	29	8.730	5.369×10^{8}
10	3.010	1.024×10^{3}	30	9.031	1.074×10^{9}
11.000	3.311	2.048×10^{3}	31.000	9.332	2.147×10^{9}
12	3.612	4.096×10^{3}	32	9.633	4.295×10^{9}
13	3.913	8.192×10^{3}	33	9.934	8.590×10^{9}
14	4.214	1.638×10^{4}	34	10.069	1.718×10^{10}
15	4.515	3.277×10^{4}	35	10.536	3.436×10^{10}
16.000	4.816	6.554×10^{4}	36.000	10.837	6.872×10^{10}
17	5.118	1.311×10^{5}	37	11.138	1.374×10^{11}
18	5.419	2.621×10^{5}	38	11.439	2.749×10^{11}
19	5.720	5.243×10^{5}	39	11.740	5.498×10^{11}
20	6.021	1.049×10^{6}	40	12.041	1.100×10^{12}

without especially increasing their usefulness, since measurements of population density are rarely sensitive enough to justify such mathematical precision. A third place may be estimated by interpolation, however. In Table II, $\log_{10}$ value 0.615 may be considered approximately halfway between the tabular values:

$$\log_{10} N = 0.610; \log_2 N = 1.026$$

and

$$\log_{10} N = 0.620; \log_2 N = 1.060$$

so that when

$$\log_{10} N = 0.615, \log_2 N = 1.043.$$

TABLE IV

CONVERSION FROM BASE 2 TO BASE 10 LOGARITHMS

	$\text{Log}_{10} N$									
$\text{Log}_2 N$	0	1	2	3	4	5	6	7	8	9
0.00	0.000	0.003	0.006	0.009	0.012	0.015	0.018	0.021	0.024	0.027
0.10	0.030	0.033	0.036	0.039	0.042	0.045	0.048	0.051	0.054	0.057
0.20	0.060	0.063	0.066	0.069	0.072	0.075	0.078	0.081	0.084	0.087
0.30	0.090	0.093	0.096	0.099	0.102	0.105	0.108	0.111	0.114	0.117
0.40	0.120	0.123	0.126	0.129	0.132	0.135	0.138	0.141	0.144	0.148
0.50	0.151	0.154	0.157	0.160	0.163	0.166	0.169	0.172	0.175	0.178
0.60	0.181	0.184	0.187	0.190	0.193	0.196	0.199	0.202	0.205	0.208
0.70	0.211	0.214	0.217	0.220	0.223	0.226	0.229	0.232	0.235	0.238
0.80	0.241	0.244	0.247	0.250	0.253	0.256	0.259	0.262	0.265	0.268
0.90	0.271	0.274	0.277	0.280	0.283	0.286	0.289	0.292	0.295	0.298

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Notes to Contributors

Manuscripts

(i) ***General***

Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should not be used. Numerous small tables should be avoided.

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All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

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Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil **(do NOT use typewriting)**, and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

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